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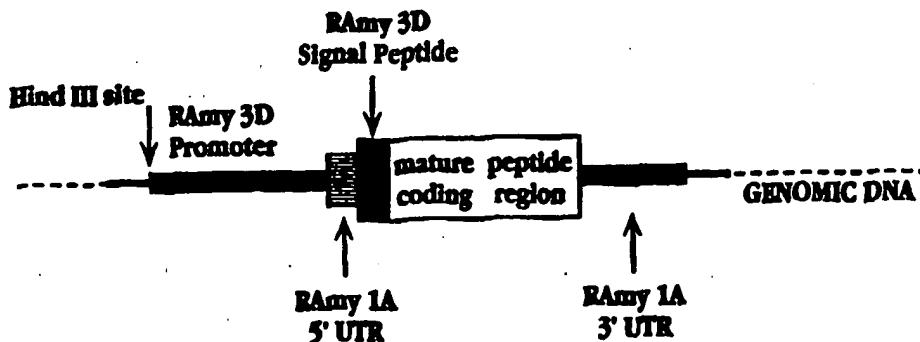
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ :		A1	(11) International Publication Number: WO 98/36085
C12N 15/82, 15/57, 15/15, 15/14, C12P 21/02			(43) International Publication Date: 20 August 1998 (20.08.98)
(21) International Application Number: PCT/US98/03068		(72) Inventors; and	
(22) International Filing Date: 13 February 1998 (13.02.98)		(75) Inventors/Applicants (for US only): SUTLIFF, Thomas, D. [US/US]; 3405 Cook Street, Rocklin, CA 94765 (US). RODRIGUEZ, Raymond, L. [US/US]; 3017 Anza Boulevard, Davis, CA 95616 (US).	
(30) Priority Data:		(74) Agents: PETITHORY, Joanne, R. et al.; Dehlinger & Associates, LLP, P.O. Box 60850, Palo Alto, CA 94306-0850 (US).	
60/038,169	13 February 1997 (13.02.97)	US	(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).
60/037,991	13 February 1997 (13.02.97)	US	
60/038,170	13 February 1997 (13.02.97)	US	
60/038,168	13 February 1997 (13.02.97)	US	
(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications		(71) Applicant (for all designated States except US): APPLIED PHYTOLOGICS, INC. [US/US]; 4110 North Freeway Boulevard, Sacramento, CA 95834-1219 (US).	
US	60/038,169 (CIP)		Published
Filed on	13 February 1997 (13.02.97)		With international search report.
US	60/037,991 (CIP)		Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.
Filed on	13 February 1997 (13.02.97)		
US	60/038,170 (CIP)		
Filed on	13 February 1997 (13.02.97)		
US	60/038,168 (CIP)		
Filed on	13 February 1997 (13.02.97)		

(54) Title: PRODUCTION OF MATURE PROTEINS IN PLANTS



(57) Abstract

A method for producing one of the following proteins in transgenic monocot plant cells is disclosed: (i) mature, glycosylated α_1 -antitrypsin (AAT) having the same N-terminal amino acid sequence as mature AAT produced in humans and a glycosylation pattern which increases serum halflife substantially over that of mature non-glycosylated AAT; (ii) mature, glycosylated antithrombin III (ATIII) having the same N-terminal amino acid sequence as mature ATIII produced in humans; (iii) mature human serum albumin (HSA) having the same N-terminal amino acid sequence as mature HSA produced in humans and having the folding pattern of native mature HSA as evidenced by its bilirubin-binding characteristics; and (iv) mature, active subtilisin BPN' (BPN') having the same N-terminal amino acid sequence as BPN' produced in *Bacillus*. Monocot plants cells are transformed with a chimeric gene which includes a DNA coding sequence encoding a fusion protein having an (i) N-terminal moiety corresponding to a rice α -amylase signal sequence peptide and, (iii) immediately adjacent the C-terminal amino acid of said peptide, a protein moiety corresponding to the mature protein to be produced.

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Production of Mature Proteins in Plants

Field of the Invention

The present invention relates to the production of mature proteins in plant cells, and in particular, to the production of proteins in mature secreted form.

5

Background of the Invention

A major commercial focus of biotechnology is the recombinant production of proteins, including both industrial enzymes and proteins that have important therapeutic uses.

Therapeutic proteins are commonly produced recombinantly by microbial expression systems, such as in *E. coli* and the yeast system *S. cerevisiae*. To date, the cost of recombinant proteins produced in a microbial host has limited the availability of a variety of therapeutically important proteins, such as human serum albumin (HSA) and α_1 -antitrypsin (AAT), to the extent that the proteins are in short supply.

Some therapeutic proteins appear to rely on glycosylation for optimal activity or stability, and the general inability of microbial systems to glycosylate or properly glycosylate mammalian proteins has also limited the usefulness of these recombinant expression systems. In some cases, proper protein folding cannot take place, because of the need for mammalian-specific foldases or other folding conditions.

To some extent, protein expression in cultured mammalian cells, or in transgenic animals may overcome the limitations of microbial expression systems. However, the cost per weight ratio of the protein is still high in mammalian expression systems, and the risk of protein contamination by mammalian viruses may be a significant regulatory problem. Protein production by transgenic animals also carries the risk of genetic variation from one generation to another. The attendant risk is variation in the recombinant protein produced, for example, variation in protein processing to yield a nature active protein with different N-terminal residue.

It would therefore be desirable to produce selected therapeutic and industrial proteins in a protein expression system that largely overcomes problems associated with microbial and mammalian-cell systems. In particular, production of the proteins should allow large volume production at low cost, and yield properly processed and glycosylated proteins. The production system should also have a relatively stable genotype from generation to generation. These aims are achieved, in the present invention, for the therapeutic proteins AAT, HSA, and antithrombin III (ATIII), and the industrial enzyme subtilisin BPN'.

Human α_1 -antitrypsin

Human α_1 -antitrypsin (AAT) is a monomer with a molecular weight of about 52Kd. Normal AAT contains 394 residues, with three complex oligosaccharide units exposed to the surface of the molecule, linked to asparagines 46, 83, and 247 (Carrell, P., *et al.*, *Nature* (1982) 298:329).

AAT is the major plasma proteinase inhibitor whose primary function is to control the proteolytic activity of trypsin, elastase, and chymotrypsin in plasma. In particular, the protein is a potent inhibitor of neutrophil elastase, and a deficiency of AAT has been observed in a number of patients with chronic emphysema of the lungs. A proportion of individuals with serum deficiency of AAT may progress to cirrhosis and liver failure (e.g., Wu, Y., *et al.*, *BioEssays* 13(4):163 (1991)).

Because of the key role of AAT as an elastase inhibitor, and because of the prevalence of genetic diseases resulting in deficient serum levels of AAT, there has been an active interest in recombinant synthesis of AAT, for human therapeutic use. To date, this approach has not been satisfactory for AAT produced by recombinant methods, for the reasons discussed above.

Human Antithrombin III

Antithrombin III (ATIII) is the major inhibitor of thrombin and factor Xa, and to a lesser extent, other serine proteases generated during the coagulation process, e.g., factors IXa, XIa, and XIIa. The inhibitory effect of ATIII is accelerated dramatically by heparin. In patients with a history of deep vein thrombosis and pulmonary embolism, the prevalence of ATIII deficiency is 2-3%.

ATIII protein has been useful in treating hereditary ATIII deficiency and has wide clinical applications for the prevention of thrombosis in high risk situations, such as surgery and delivery, and for treating acute thrombotic episodes, when used in combination with heparin.

ATIII is a glycoprotein with a molecular weight of 58,200, having 432 amino acids and containing three disulfide linkages and four asparagine-linked biantennary carbohydrate chains. Because of the key role of ATIII as an anti-thrombotic agent, and because of the broad clinical potential in anti-thrombosis therapy, there has been an active interest in recombinant synthesis of ATIII, for human therapeutic use. To date, this approach has not been satisfactory for ATIII produced by microbial or mammalian recombinant methods, for the reasons discussed above.

Human Serum Albumin

Serum albumin is the main protein component of plasma. Its main function is regulation of colloidal osmotic pressure in the bloodstream. Serum albumin binds numerous ions and small molecules, including Ca^{2+} , Na^+ , K^+ , fatty acids, hormones, bilirubin and certain drugs.

Human serum albumin (HSA) is expressed as a 609 amino acid prepro-protein which is further processed by removal of an amino-terminal peptide and an additional six amino acid residues to form the mature protein. The mature protein found in human serum is a monomeric, unglycosylated protein 585 amino acids in length (66 kDa), with a globular structure maintained by 5 17 disulfide bonds. The pattern of disulfide links forms a structural unit of one small and two large disulfide-linked double loops (Geisow, M.J. et al. (1977) Biochem. J. 163:477-484) which forms a high-affinity bilirubin binding site.

HSA is used to expand blood volume and raise low blood protein levels in cases of shock, trauma, and post-surgical recovery. HSA is often administered in emergency situations to stabilize 10 blood pressure.

Because of the key role of HSA as an osmotic stabilizing agent, and because of its broad clinical potential in, e.g., plasma replacement therapy, there has been an active interest in recombinant synthesis of HSA for human therapeutic use. This approach has not been satisfactory for HSA produced by microbial or mammalian recombinant methods, for the reasons discussed 15 above.

Subtilisin BPN'

Subtilisin BPN' (BPN') is an important industrial enzyme, particularly for use as a detergent enzyme. Several groups have reported amino acid substitution modifications of the 20 enzyme that are effective in enhancing the activity, pH optimum, stability and/or therapeutic use of the enzyme.

BPN' is expressed in as a 381 amino acid preproenzyme, including 35 amino acid sequence required for secretion and a 77 amino acid moiety which serves as a chaperon to facilitate folding. Studies indicate that the pro moiety acts in trans outside of cells.

To date, large-scale production of BPN' is predominantly by microbial fermentation, which 25 has relatively high costs associated with it. In addition, the enzyme tends to auto-degrade at optimal fermentation growth-medium conditions.

Summary of the Invention

In one aspect, the invention includes a method of producing, in monocot plant cells, a 30 mature heterologous protein selected from the group consisting of (i) mature, glycosylated α_1 -antitrypsin (AAT) having the same N-terminal amino acid sequence as mature AAT produced in humans and a glycosylation pattern which increases serum halflife substantially over that of non-glycosylated mature AAT; (ii) mature, glycosylated antithrombin III (ATIII) having the same N-terminal amino acid sequence as mature ATIII produced in humans; (iii) mature human serum 35 albumin (HSA) having the same N-terminal amino acid sequence as mature HSA produced in

humans and having the folding pattern of native mature HSA as evidenced by its bilirubin-binding characteristics; and (iv) mature, active subtilisin BPN' (BPN'), glycosylated or non-glycosylated, having the same N-terminal amino acid sequence as BPN' produced in *Bacillus*.

The method includes obtaining monocot cells transformed with a chimeric gene having (i) a 5 monocot transcriptional regulatory region, inducible by addition or removal of a small molecule, or during seed maturation, (ii) a first DNA sequence encoding the heterologous protein, and (iii) a second DNA sequence encoding a signal peptide. The second DNA sequence is operably linked to the transcriptional regulatory region and to the first DNA sequence. The first DNA sequence is in translation-frame with the second DNA sequence, and the two sequences encode a fusion protein. 10 The transformed cells are cultivated under conditions effective to induce the transcriptional regulatory region, thereby promoting expression of the fusion protein and secretion of the mature heterologous protein from the transformed cells. The mature heterologous protein produced by the transformed cells is then isolated.

In one embodiment of the method, the first DNA sequence encodes pro-subtilisin BPN' 15 (proBPN'), the cultivating includes cultivating the transformed cells at a pH between 5 and 6, and the isolating step includes incubating the proBPN' to under condition effective to allow its autoconversion to active mature BPN'. In another embodiment, the first DNA sequence encodes mature BPN', and the cells are transformed with a second chimeric gene containing (i) a transcriptional regulatory region inducible by addition or removal of a small molecule, (ii) a third DNA 20 sequence encoding the pro-peptide moiety of BPN', and (iii) a fourth DNA sequence encoding a signal polypeptide. The fourth DNA sequence is operably linked to the transcriptional regulatory region and to the third DNA sequence, and the signal polypeptide is in translation-frame with the pro-peptide moiety and is effective to facilitate secretion of expressed pro-peptide moiety from the transformed cells. The cultivating step includes cultivating the transformed cells at a pH between 5 25 and 6, and the isolating step includes incubating the mature BPN' and the pro-moiety under conditions effective to allow the conversion of BPN' by the pro- moiety to active mature BPN'.

In another embodiment of the method, the signal peptide is the RAmy3D signal peptide (SEQ ID NO:1) or the RAmy1A signal peptide (SEQ ID NO:4). The coding sequence of the signal peptide may be a codon-optimized sequence, such as the codon-optimized RAmy3D sequence 30 identified as SEQ ID NO:3. The first DNA sequence may also be codon-optimized. Exemplary codon-optimized signal peptide-heterologous protein fusion protein coding sequences include 3D-AAT (SEQ ID NO:18), 3D-ATIII (SEQ ID NO:19), and 3D-HSA (SEQ ID NO:20). The first DNA sequence may further contain codon substitutions which eliminate one or more potential glycosylation sites present in the native amino acid sequence of the heterologous protein, such as the 35 codon-optimized sequence encoding 3D-proBPN' (SEQ ID NO:21).

In other embodiments of the method, the transcriptional regulatory region may be a promoter derived from a rice or barley α -amylase gene, including RAmy1A, RAmy1B, RAmy2A, RAmy3A, RAmy3B, RAmy3C, RAmy3D, RAmy3E, pM/C, gKAmy141, gKAmy155, Amy32b, or HV18. The chimeric gene may further include, between the transcriptional regulatory region and the 5 fusion protein coding sequence, the 5' untranslated region (5' UTR) of an inducible monocot gene such as one of the rice or barley α -amylase genes described above. One preferred 5' UTR is that from the RAmy1A gene, which is effective to enhance the stability of the gene transcript. The chimeric gene may further include, downstream of the coding sequence, the 3' untranslated region (3' UTR) from an inducible monocot gene, such as one of the rice or barley α -amylase genes 10 mentioned above. One preferred 3' UTR is from the RAmy1A gene.

Where the method is employed in protein production in a monocot cell culture, preferred promoters are the RAmy3D and RAmy3E gene promoters, which are upregulated by sugar depletion in cell culture. Where the gene is employed in protein production in germinating seeds, a preferred promoter is the RAmy1A gene promoter, which is upregulated by gibberellic acid during 15 seed germination. Where gene is upregulated during seed maturation, a preferred promoter is the barley endosperm-specific B1-hordein promoter.

The invention also includes a mature heterologous protein produced by the above method. The protein has a glycosylation pattern characteristic of the monocot plant in which the protein is produced. The glycosylated protein is selected from the group consisting of (i) mature glycosylated 20 α_1 -antitrypsin (AAT) having the same N-terminal amino acid sequence as mature AAT produced in humans and having a glycosylation pattern which increases serum halflife substantially over that of non-glycosylated mature AAT; (ii) mature glycosylated antithrombin III (ATIII) having the same N-terminal amino acid sequence as mature ATIII produced in humans; and (iii) mature glycosylated subtilisin BPN' (BPN') having the same N-terminal amino acid sequence as BPN' produced in 25 *Bacillus*.

The invention also includes plant cells and seeds capable of producing the mature heterologous proteins according to the above method.

These and other objects and features of the invention will be more fully understood when the following detailed description of the invention is read in conjunction with the accompanying 30 drawings.

Brief Description of the Figures

Fig. 1 shows, in the lower row, the amino acid sequence of a RAmy3D signal sequence portion employed in the invention, identified as SEQ ID NO:1; in the middle row, the corresponding native coding sequence, identified as SEQ ID NO:2; and in the upper row, a 35 corresponding codon-optimized sequence, identified as SEQ ID NO:3;

Fig. 2 illustrates the components of a chimeric gene constructed in accordance with an embodiment of the invention;

Figs. 3A and 3B illustrate the construction of an exemplary transformation vector for use in transforming a monocot plant, for production of a mature protein in cell culture in accordance with 5 one embodiment of the invention (native mature AAT coding sequence under control of the RAmy3D promoter and signal sequence);

Fig. 4 illustrates factors in the metabolic regulation of AAT production in rice cell culture;

Fig. 5 shows immunodetection of AAT using antibody raised against the C-terminal region of AAT;

10 Fig. 6 shows Western blot analysis of AAT produced by transformed rice cell lines 18F, 11B, and 27F;

Fig. 7 shows the time course of elastase:AAT complex formation in human and rice-produced forms of AAT;

15 Fig. 8 shows an N-terminal sequence for mature α_1 -antitrypsin (AAT) produced in accordance with the invention, identified herein as SEQ ID NO:22;

Fig. 9 shows a Western blot of ATIII produced in accordance with the invention;

Fig. 10 shows a Western blot of plant-produced BPN', comparing expression from codon-optimized and native coding sequences;

20 Fig. 11 compares the specific activity of BPN' codon-optimized (AP106) vs. BPN' native (AP101) expression in rice callus cell culture; and

Fig. 12 shows a western blot of HSA produced in germinating seeds in accordance with the invention.

Brief Description of the Sequences

25 SEQ ID NO:1 is the amino acid sequence of the RAmy3D signal peptide;

SEQ ID NO:2 is the native sequence encoding the RAmy3D signal peptide;

SEQ ID NO:3 is a codon-optimized sequence encoding the RAmy3D signal peptide;

SEQ ID NO:4 is the amino acid sequence of the RAmy1A signal peptide;

SEQ ID NO:5 is the 5' UTR derived from the RAmy1A gene;

30 SEQ ID NO:6 is the 3' UTR derived from the RAmy1A gene;

SEQ ID NO:7 is the amino acid sequence of mature α_1 -antitrypsin (AAT);

SEQ ID NO:8 is the native DNA coding sequence of mature AAT;

SEQ ID NO:9 is the amino acid sequence of mature antithrombin III (ATIII);

SEQ ID NO:10 is the native DNA coding sequence of mature ATIII;

35 SEQ ID NO:11 is the amino acid sequence of mature human serum albumin (HSA);

SEQ ID NO:12 is the native DNA coding sequence of mature HSA;
SEQ ID NO:13 is the amino acid sequence of native proBPN';
SEQ ID NO:14 is the native DNA coding sequence of proBPN';
SEQ ID NO:15 is the amino acid sequence of the "pro" moiety of BPN';
5 SEQ ID NO:16 is the amino acid sequence of native mature BPN';
SEQ ID NO:17 is the amino acid sequence of a mature BPN' variant in which all potential N-glycosylation sites are removed according to Table 2;
SEQ ID NO:18 is a codon-optimized sequence encoding the RAmy3D signal sequence/mature α_1 -antitrypsin fusion protein;
10 SEQ ID NO:19 is a sequence encoding the RAmy3D signal sequence/mature antithrombin III fusion protein, with a codon-optimized RAmy3D coding sequence fused to the native mature ATIII coding sequence;
SEQ ID NO:20 is a sequence encoding the RAmy3D signal sequence/mature human serum albumin fusion protein, with a codon-optimized RAmy3D coding sequence fused to the native 15 mature HSA coding sequence;
SEQ ID NO:21 is a codon-optimized sequence encoding the RAmy3D signal sequence/prosubtilisin BPN' fusion protein;
SEQ ID NO:22 is the N-terminal sequence of mature α_1 -antitrypsin produced in accordance with the invention;
20 SEQ ID NO:23 is an oligonucleotide used to prepare the intermediate p3DProSig construct of Example 1;
SEQ ID NO:24 is the complement of SEQ ID NO:23;
SEQ ID NO:25 is an oligonucleotide used to prepare the intermediate p3DProSigENDlink construct of Example 1;
25 SEQ ID NO:26 is the complement of SEQ ID NO:25;
SEQ ID NO:27 is one of six oligonucleotides used to prepare the intermediate p1AProSig construct of Example 1;
SEQ ID NO:28 is one of six oligonucleotides used to prepare the intermediate p1AProSig construct of Example 1;
30 SEQ ID NO:29 is one of six oligonucleotides used to prepare the intermediate p1AProSig construct of Example 1;
SEQ ID NO:30 is one of six oligonucleotides used to prepare the intermediate p1AProSig construct of Example 1;
35 SEQ ID NO:31 is one of six oligonucleotides used to prepare the intermediate p1AProSig construct of Example 1;

SEQ ID NO:32 is one of six oligonucleotides used to prepare the intermediate p1AProSig construct of Example 1;

SEQ ID NO:33 is the N-terminal primer used to PCR-amplify the AAT coding sequence according to Example 1; and

5 SEQ ID NO:34 is the C-terminal primer used to PCR-amplify the AAT coding sequence according to Example 1.

Detailed Description of the Invention

I. Definitions:

10 The terms below have the following meaning, unless indicated otherwise in the specification.

"Cell culture" refers to cells and cell clusters, typically callus cells, growing on or suspended in a suitable growth medium.

15 "Germination" refers to the breaking of dormancy in a seed and the resumption of metabolic activity in the seed, including the production of enzymes effective to break down starches in the seed endosperm.

"Inducible" means a promoter that is upregulated by the presence or absence of a small molecules. It includes both indirect and direct inducement.

20 "Inducible during germination" refers to promoters which are substantially silent but not totally silent prior to germination but are turned on substantially (greater than 25%) during germination and development in the seed. Examples of promoters that are inducible during germination are presented below.

25 "Small molecules", in the context of promoter induction, are typically small organic or bioorganic molecules less than about 1 kDa. Examples of such small molecules include sugars, sugar-derivatives (including phosphate derivatives), and plant hormones (such as, gibberellic or abscisic acid).

30 "Specifically regulatable" refers to the ability of a small molecule to preferentially affect transcription from one promoter or group of promoters (e.g., the α -amylase gene family), as opposed to non-specific effects, such as, enhancement or reduction of global transcription within a cell by a small molecule.

35 "Seed maturation" or "grain development" refers to the period starting with fertilization in which metabolizable reserves, e.g., sugars, oligosaccharides, starch, phenolics, amino acids, and proteins, are deposited, with and without vacuole targeting, to various tissues in the seed (grain), e.g., endosperm, testa, aleurone layer, and scutellar epithelium, leading to grain enlargement, grain filling, and ending with grain desiccation.

"Inducible during seed maturation" refers to promoters which are turned on substantially (greater than 25%) during seed maturation.

Heterologous DNA" or "foreign DNA" refers to DNA which has been introduced into plant cells from another source, or which is from a plant source, including the same plant source, 5 but which is under the control of a promoter or terminator that does not normally regulate expression of the heterologous DNA.

"Heterologous protein" is a protein, including a polypeptide, encoded by a heterologous DNA. A "transcription regulatory region" or "promoter" refers to nucleic acid sequences that influence and/or promote initiation of transcription. Promoters are typically considered to include 10 regulatory regions, such as enhancer or inducer elements.

A "chimeric gene," in the context of the present invention, typically comprises a promoter sequence operably linked to DNA sequence that encodes a heterologous gene product, e.g., a selectable marker gene or a fusion protein gene. A chimeric gene may also contain further transcription regulatory elements, such as transcription termination signals, as well as translation 15 regulatory signals, such as, termination codons.

"Operably linked" refers to components of a chimeric gene or an expression cassette that function as a unit to express a heterologous protein. For example, a promoter operably linked to a heterologous DNA, which encodes a protein, promotes the production of functional mRNA corresponding to the heterologous DNA.

20 A "product" encoded by a DNA molecule includes, for example, RNA molecules and polypeptides.

"Removal" in the context of a metabolite includes both physical removal as by washing and the depletion of the metabolite through the absorption and metabolizing of the metabolite by the cells.

25 "Substantially isolated" is used in several contexts and typically refers to the at least partial purification of a protein or polypeptide away from unrelated or contaminating components. Methods and procedures for the isolation or purification of proteins or polypeptides are known in the art.

30 "Stably transformed" as used herein refers to a cereal cell or plant that has foreign nucleic acid stably integrated into its genome which is transmitted through multiple generations.

" α_1 -antitrypsin or "AAT" refers to the protease inhibitor which has an amino acid sequence substantially identical or homologous to AAT protein identified by SEQ ID NO:7.

35 "Antithrombin III" or "ATIII" refers to the heparin-activated inhibitor of thrombin and factor Xa, and which has an amino acid sequence substantially identical or homologous to ATIII protein identified by SEQ ID NO:9.

"Human serum albumin" or "HSA" refers to a protein which has an amino acid sequence substantially identical or homologous to the mature HSA protein identified by SEQ ID NO:11.

"Subtilisin" or "subtilisin BPN'" or "BPN'" refers to the protease enzyme produced naturally by *B. amyloliquefaciens*, and having the sequence of SEQ ID NO:16, or a sequence 5 homologous therewith.

"proBPN'" refers to a form of BPN' having an approximately 78 amino-acid "pro" moiety that functions as a chaperon polypeptide to assist in folding and activation of the BPN', and having the sequence in SEQ ID NO:13, or a sequence homologous therewith.

"Codon optimization" refers to changes in the coding sequence of a gene to replace native 10 codons with those corresponding to optimal codons in the host plant.

A DNA sequence is "derived from" a gene, such as a rice or barley α -amylase gene, if it corresponds in sequence to a segment or region of that gene. Segments of genes which may be derived from a gene include the promoter region, the 5' untranslated region, and the 3' untranslated region of the gene.

15

II. Transformed plant cells

The plants used in the process of the present invention are derived from monocots, particularly the members of the taxonomic family known as the Gramineae. This family includes all members of the grass family of which the edible varieties are known as cereals. The cereals include 20 a wide variety of species such as wheat (*Triticum ssp.*), rice (*Oryza ssp.*) barley (*Hordeum ssp.*) oats, (*Avena ssp.*) rye (*Secale ssp.*), corn (*Zea ssp.*) and millet (*Pennisetum ssp.*). In the present invention, preferred family members are rice and barley.

Plant cells or tissues derived from the members of the family are transformed with expression constructs (*i.e.*, plasmid DNA into which the gene of interest has been inserted) using a 25 variety of standard techniques (*e.g.*, electroporation, protoplast fusion or microparticle bombardment). The expression construct includes a transcription regulatory region (promoter) whose transcription is specifically upregulated by the presence of absence of a small molecule, such as the reduction or depletion of sugar, *e.g.*, sucrose, in culture medium, or in plant tissues, *e.g.*, germinating seeds. In the present invention, particle bombardment is the preferred transformation 30 procedure.

The construct also includes a gene encoding a mature heterologous protein in a form suitable for secretion from plant cells. The gene encoding the recombinant heterologous protein is placed under the control of a metabolically regulated promoter. Metabolically regulated promoters are those in which mRNA synthesis or transcription, is repressed or upregulated by a small 35 metabolite or hormone molecule, such as the rice RAmy3D and RAmy3E promoters, which are

upregulated by sugar-depletion in cell culture. For protein production in germinating seeds from regenerated transgenic plants, a preferred promoter is the Ramy 1A promoter, which is up-regulated by gibberellic acid during seed germination. The expression construct also utilizes additional regulatory DNA sequences *e.g.*, preferred codons, termination sequences, to promote efficient
5 translation of AAT, as will be described.

A. Plant Expression Vector

Expression vectors for use in the present invention comprise a chimeric gene (or expression cassette), designed for operation in plants, with companion sequences upstream and downstream
10 from the expression cassette. The companion sequences will be of plasmid or viral origin and provide necessary characteristics to the vector to permit the vectors to move DNA from bacteria to the desired plant host. Suitable transformation vectors are described in related application PCT WO 95/14099, published May 25, 1995, which is incorporated by reference herein. Suitable components of the expression vector, including an inducible promoter, coding sequence for a signal
15 peptide, coding sequence for a mature heterologous protein, and suitable termination sequences are discussed below. One exemplary vector is the p3D(AAT)v1.0 vector illustrated in Figs 3A and 3B.

A1. Promoters

The transcription regulatory or promoter region is chosen to be regulated in a manner allowing for induction under selected cultivation conditions, *e.g.*, sugar depletion in culture or water uptake followed by gibberellic acid production in germinating seeds. Suitable promoters, and their method of selection are detailed in above-cited PCT application WO 95/14099. Examples of such promoters include those that transcribe the cereal α -amylase genes and sucrose synthase genes, and are repressed or induced by small molecules, like sugars, sugar depletion or phytohormones
25 such as gibberellic acid or abscisic acid. Representative promoters include the promoters from the rice α -amylase RAmy1A, RAmy1B, RAmy2A, RAmy3A, RAmy3B, RAmy3C, RAmy3D, and RAmy3E genes, and from the pM/C, gKAmy141, gKAmy155, Amy32b, and HV18 barley α -amylase genes. These promoters are described, for example, in ADVANCES IN PLANT BIOTECHNOLOGY Ryu, D.D.Y., *et al*, Eds., Elsevier, Amsterdam, 1994, p.37, and references cited
30 therein. Other suitable promoters include the sucrose synthase and sucrose-6-phosphate-synthetase (SPS) promoters from rice and barley.

Other suitable promoters include promoters which are regulated in a manner allowing for induction under seed-maturation conditions. Examples of such promoters include those associated with the following monocot storage proteins: rice glutelins, oryzins, and prolamines, barley
35 hordeins, wheat gliadins and glutelins, maize zeins and glutelins, oat glutelins, and sorghum

kafirins, millet pennisetins, and rye secalins.

A preferred promoter for expression in germinating seeds is the rice α -amylase RAmy1A promoter, which is upregulated by gibberellic acid. Preferred promoters for expression in cell culture are the rice α -amylase RAmy3D and RAmy3E promoters which are strongly upregulated by sugar depletion in the culture. These promoters are also active during seed germination. A preferred promoter for expression in maturing seeds is the barley endosperm-specific B1-hordein promoter (Brandt, A., et al., (1985) Carlsberg Res. Commun. 50:333-345).

The chimeric gene may further include, between the promoter and coding sequences, the 5' untranslated region (5' UTR) of an inducible monocot gene, such as the 5' UTR derived from one of the rice or barley α -amylase genes mentioned above. One preferred 5' UTR is that derived from the RAmy1A gene, which is effective to enhance the stability of the gene transcript. This 5' UTR has the sequence given by SEQ ID NO:5 herein.

A2. Signal Sequences

In addition to encoding the protein of interest, the chimeric gene encodes a signal sequence (or signal peptide) that allows processing and translocation of the protein, as appropriate. Suitable signal sequences are described in above-referenced PCT application WO 95/14099. One preferred signal sequence is identified as SEQ ID NO:1 and is derived from the RAmy3D promoter. Another preferred signal sequence is identified as SEQ ID NO:4 and is derived from the RAmy1A promoter. The plant signal sequence is placed in frame with a heterologous nucleic acid encoding a mature protein, forming a construct which encodes a fusion protein having an N-terminal region corresponding to the signal peptide and, immediately adjacent to the C-terminal amino acid of the signal peptide, the N-terminal amino acid of the mature heterologous protein. The expressed fusion protein is subsequently secreted and processed by signal peptidase cleavage precisely at the junction of the signal peptide and the mature protein, to yield the mature heterologous protein.

In another embodiment of the invention, the coding sequence in the fusion protein gene, in at least the coding region for the signal sequence, may be codon-optimized for optimal expression in plant cells, e.g., rice cells, as described below. The upper row in Fig. 1 shows one codon-optimized coding sequence for the RAmy3D signal sequence, identified herein as SEQ ID NO:3.

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A3. Naturally-Ocurring Heterologous Protein Coding Sequences

(i) α_1 -Antitrypsin: Mature human AAT is composed of 394 amino acids, having the sequence identified herein as SEQ ID NO:7. The protein has N-glycosylation sites at asparagines 46, 83 and 247. The corresponding native DNA coding sequence is identified herein as SEQ ID NO:8.

(ii) Antithrombin III: Mature human ATIII is composed of 432 amino acids, having the sequence identified herein as SEQ ID NO:9. The protein has N-glycosylation sites at the four asparagine residues 96, 135, 155, and 192. The corresponding native DNA coding sequence is identified herein as SEQ ID NO:10.

5 (iii) Human serum albumin: Mature HSA as found in human serum is composed of 585 amino acids, having the sequence identified herein as SEQ ID NO:11. The protein has no N-linked glycosylation sites. The corresponding native DNA coding sequence is identified herein as SEQ ID NO:12.

10 (iv) Subtilisin BPN': Native proBPN' as produced in *B. amyloliquefaciens* is composed of 352 amino acids, having the sequence identified herein as SEQ ID NO:13. The corresponding native DNA coding sequence is identified herein as SEQ ID NO:14. The proBPN' polypeptide contains a 77 amino acid "pro" moiety which is identified herein as SEQ ID NO:15. The remainder of the polypeptide, which forms the mature active BPN', is a 275 amino acid sequence identified herein by SEQ ID NO:16. Native BPN' as produced in *Bacillus* is not glycosylated.

15

A4. Codon-Optimized Coding Sequences

In accordance with one aspect of the invention, it has been discovered that a severalfold enhancement of expression level can be achieved in plant cell culture by modifying the native coding sequence of a heterologous gene by contain predominantly or exclusively, highest-frequency 20 codons found in the plant cell host.

The method will be illustrated for expression of a heterologous gene in rice plant cells, it being recognized that the method is generally applicable to any monocot. As a first step, a representative set of known coding gene sequence from rice is assembled. The sequences are then analyzed for codon frequency for each amino acid, and the most frequent codon is selected for each 25 amino acid. This approach differs from earlier reported codon matching methods, in which more than one frequent codon is selected for at least some of the amino acids. The optimal codons selected in this manner for rice and barley are shown in Table 1.

Table 1

Amino Acid	Rice Preferred Codon	Barley Preferred Codon
Ala A	GCC	
Arg R	CGC	
Asn N	AAC	

30

Amino Acid	Rice Preferred Codon	Barley Preferred Codon
Asp D	GAC	
Cys C	UGC	
Gln Q	CAG	
Glu E	GAG	
Gly G	GGC	
His H	CAC	
Ile I	AUC	
Leu L	CUC	
Lys K	AAG	
Phe F	UUC	
Pro P	CCG	CCC
Ser S	AGC	UCC
Thr T	ACC	
Tyr Y	UAC	
Val V	GUC	GUG
stop	UAA	UGA

As indicated above, the fusion protein coding sequence in the chimeric gene is constructed such that the final (C-terminal) codon in the signal sequence is immediately followed by the codon 5 for the N-terminal amino acid in the mature form of the heterologous protein. Exemplary fusion protein genes, in accordance with the present invention, are identified herein as follows:

SEQ ID NO:18, corresponding to codon-optimized coding sequences of the fusion protein consisting of RAmy3D signal sequence/mature α_1 -antitrypsin;

SEQ ID NO:19, corresponding to the fusion protein coding sequence consisting of the 10 codon-optimized RAmy3D signal sequence and the native mature antithrombin III sequence;

SEQ ID NO:20, corresponding to the fusion protein coding sequence consisting of the codon-optimized RAmy3D signal sequence and the native mature human serum albumin sequence;

SEQ ID NO:21, corresponding to codon-optimized coding sequence of the fusion protein RAmy3D signal sequence/prosubtilisin BPN'. In this instance, prosubtilisin is considered the 15 "mature" protein, in that secreted prosubtilisin can autocatalyze to active, mature subtilisin.

In a preferred embodiment, the BPN' coding sequence is further modified to eliminate

potential N-glycosylation sites, as native BPN' is not glycosylated. Table 2 illustrates preferred codon substitutions, which eliminate all potential N-glycosylation sites in subtilisin BPN'. SEQ ID NO:17 corresponds to a mature BPN' amino acid sequence containing the substitutions presented in Table 2.

5

Table 2

N-Glycosylation Sites	Location (Asn) (in mature protein)	Amino Acid Substitution
Asn Asn Ser	61	Thr Asn Ser
Asn Asn Ser	76	Thr Asn Ser
Asn Met Ser	123	Thr Met Ser
Asn Gly Thr	218	Ser Gly Thr ¹
Asn Trp Thr	240	Thr Trp Thr

¹improved thermostability; Bryan, *et al.*, *Proteins: Structure, Function, and Genetics* 1:326 (1986).

10

A5. Transcription and Translation Terminators

The chimeric gene may also include, downstream of the coding sequence, the 3' untranslated region (3' UTR) from an inducible monocot gene, such as one of the rice or barley α -amylase genes mentioned above. One preferred 3' UTR is that derived from the RAmy1A gene, whose sequence is given by SEQ ID NO:6. This sequence includes non-coding sequence 5' to the polyadenylation site, the polyadenylation site, and the transcription termination sequence. The transcriptional termination region may be selected, particularly for stability of the mRNA to enhance expression. Polyadenylation tails (Alber and Kawasaki, 1982, *Mol. and Appl. Genet.* 1:419-434) are also commonly added to the expression cassette to optimize high levels of transcription and proper transcription termination, respectively. Polyadenylation sequences include but are not limited to the *Agrobacterium* octopine synthetase signal (Gielen, *et al.*, *EMBO J.* 3:835-846 (1984) or the nopaline synthase of the same species (Depicker, *et al.*, *Mol. Appl. Genet.* 1:561-573 (1982)).

Since the ultimate expression of the heterologous protein will be in a eukaryotic cell (in this case, a member of the grass family), it is desirable to determine whether any portion of the cloned gene contains sequences which will be processed out as introns by the host's splicing machinery. If so, site-directed mutagenesis of the "intron" region may be conducted to prevent losing a portion of the genetic message as a false intron code (Reed and Maniatis, *Cell* 41:95-105 (1985)).

Fig. 2 shows the elements of one preferred chimeric gene constructed in accordance with the invention, and intended particularly for use in protein expression in a rice cell suspension culture. The gene includes, in a 5' to 3' direction, the promoter from the RAmy3D gene, which is inducible in cell culture with sugar depletion, the 5' UTR from the RAmy1A gene, which confers enhanced stability on the gene transcript, the RAmy3D signal sequence coding region, as identified above, the coding region of a heterologous protein to be produced, and a 3' UTR region from the RAmy1A gene.

III. Plant Transformation

For transformation of plants, the chimeric gene is placed in a suitable expression vector designed for operation in plants. The vector includes suitable elements of plasmid or viral origin that provide necessary characteristics to the vector to permit the vectors to move DNA from bacteria to the desired plant host. Suitable transformation vectors are described in related application PCT WO 95/14099, published May 25, 1995, which is incorporated by reference herein. Suitable components of the expression vector, including the chimeric gene described above, are discussed below. One exemplary vector is the p3Dv1.0 vector described in Example 1.

A. Transformation Vector

Vectors containing a chimeric gene of the present invention may also include selectable markers for use in plant cells (such as the *nptII* kanamycin resistance gene, for selection in kanamycin-containing or the phosphinothricin acetyltransferase gene, for selection in medium containing phosphinothricin (PPT).

The vectors may also include sequences that allow their selection and propagation in a secondary host, such as sequences containing an origin of replication and a selectable marker such as antibiotic or herbicide resistance genes, e.g., HPH (Hagio *et al.*, *Plant Cell Reports* 14:329 (1995); van der Elzer, *Plant Mol. Biol.* 5:299-302 (1985). Typical secondary hosts include bacteria and yeast. In one embodiment, the secondary host is *Escherichia coli*, the origin of replication is a colE1-type, and the selectable marker is a gene encoding ampicillin resistance. Such sequences are well known in the art and are commercially available as well (e.g., Clontech, Palo Alto, CA; Stratagene, La Jolla, CA).

The vectors of the present invention may also be modified to intermediate plant transformation plasmids that contain a region of homology to an *Agrobacterium tumefaciens* vector, a T-DNA border region from *Agrobacterium tumefaciens*, and chimeric genes or expression cassettes (described above). Further, the vectors of the invention may comprise a disarmed plant tumor inducing plasmid of *Agrobacterium tumefaciens*.

The vector described in Example 1, and having a promoter from the RAmy3D gene, is suitable for use in a method of mature protein production in cell culture, where the RAmy3D promoter is induced by sugar depletion in cell culture medium. Other promoters may be selected for other applications, as indicated above. For example, for mature protein expression in 5 germinating seeds, the coding sequence may be placed under the control of the rice α -amylase RAmy1A promoter, which is inducible by gibberellic acid during seed germination.

B. Transformation of plant cells

Various methods for direct or vectored transformation of plant cells, e.g., plant protoplast 10 cells, have been described, e.g., in above-cited PCT application WO 95/14099. As noted in that reference, promoters directing expression of selectable markers used for plant transformation (e.g., nptII) should operate effectively in plant hosts. One such promoter is the nos promoter from native Ti plasmids (Herrera-Estrella, et al., *Nature* 303:209-213 (1983). Others include the 35S and 19S 15 promoters of cauliflower mosaic virus (Odell, et al., *Nature* 313:810-812 (1985) and the 2' promoter (Velten, et al., *EMBO J.* 3:2723-2730 (1984).

In one preferred embodiment, the embryo and endosperm of mature seeds are removed to exposed scutulum tissue cells. The cells may be transformed by DNA bombardment or injection, or by vectored transformation, e.g., by *Agrobacterium* infection after bombarding the scutellum cells with microparticles to make them susceptible to *Agrobacterium* infection (Bidney et al., *Plant Mol.* 20 *Biol.* 18:301-313, 1992).

One preferred transformation follows the methods detailed generally in Sivamani, E. et al., *Plant Cell Reports* 15:465 (1996); Zhang, S., et al., *Plant Cell Reports* 15:465 (1996); and Li, L., et al., *Plant Cell Reports* 12:250 (1993). Briefly, rice seeds are sterilized by standard methods, and callus induction from the seeds is carried out on MB media with 2,4D. During a first incubation 25 period, callus tissue forms around the embryo of the seed. By the end of the incubation period, (e.g., 14 days at 28°C) the calli are about 0.25 to 0.5 cm in diameter. Callus mass is then detached from the seed, and placed on fresh NB media, and incubated again for about 14 days at 28°C. After the second incubation period, satellite calli developed around the original "mother" callus mass. These satellite calli were slightly smaller, more compact and defined than the original tissue. It was 30 these calli were transferred to fresh media. The "mother" calli was not transferred. The goal was to select only the strongest, most vigorous growing tissue for further culture.

Calli to be bombarded are selected from 14-day-old subcultures. The size, shape, color and density are all important in selecting calli in the optimal physiological condition for transformation. The calli should be between .8 and 1.1 mm in diameter. The calli should appear as spherical 35 masses with a rough exterior.

Transformation is by particle bombardment, as detailed in the references cited above. After the transformation steps, the cells are typically grown under conditions that permit expression of the selectable marker gene. In a preferred embodiment, the selectable marker gene is HPH. It is preferred to culture the transformed cells under multiple rounds of selection to produce a uniformly stable transformed cell line.

IV. Cell Culture Production of Mature Heterologous Protein

Transgenic cells, typically callus cells, are cultured under conditions that favor plant cell growth, until the cells reach a desired cell density, then under conditions that favor expression of the mature protein under the control of the given promoter. Preferred culture conditions are described below and in Example 2. Purification of the mature protein secreted into the medium is by standard techniques known by those of skill in the art.

Production of mature AAT: In a preferred embodiment, the culture medium contains a phosphate buffer, *e.g.*, the 20 mM phosphate buffer, pH 6.8 described in Example 2, to reduce AAT degradation catalyzed by metals. Alternatively, or in addition, a metal chelating agent, such as EDTA, may be added to the medium.

Following the cell culture method described in Example 2, cell culture media was partially purified and the fraction containing AAT was analyzed by Western blot, as shown in Fig. 4. The first two lanes ("phosphate") show AAT bands both in the presence and absence of elastase ("+E" and "-E"), where the higher molecular weight bands in the presence of elastase correspond roughly to a 58-59 kdal AAT/elastase complex. Also as seen in the figure, expression was high in the absence of sucrose, but nearly undetectable in the presence of sucrose.

To ascertain the degree of glycosylation (as determined by apparent molecular weight by SDS-PAGE) the protein produced in culture was fractionated by SDS-PAGE and immunodetected with a labeled antibody raised against the C-terminal portion of AAT, as shown in Fig. 5. Lane 4 contains human AAT, and its migration position corresponds to about 52 kdal. In lane 3 is the plant-produced AAT, having an apparent molecular weight of about 49-50 kdal, indicating an extent of glycosylation of up to 60-80% of the glycosylation found in human AAT (non-glycosylated AAT has a molecular weight of 45 kdal).

Similar results are shown in the Western blots in Fig. 6. Lanes 1-3 in this figure correspond to decreasing amount (15, 10, and 5 ng) of human AAT; lane 4, to 10 μ l supernatant from a non-expressing plant cell line; lanes 5 and 6, to 10 μ l supernatant from AAT-expressing plant cell lines 11B and 27F, respectively, and lane 7, to 10 μ l supernatant from cell line 27F plus 250 ng trypsin. The upward mobility shift in lane 7 is indicative of association between trypsin and the plant-produced AAT.

The ability of plant-produced AAT to bind to elastase is demonstrated in Fig. 7, which shows the shift in molecular weight over a 30 minute binding interval for the 52 kdal human AAT (lanes 1-4) and the 49-50 kdal plant-produced AAT.

To demonstrate that the mature protein is produced in secreted form, with the desired N-terminus, a chimeric gene constructed as above, and having the coding sequence for mature α_1 -antitrypsin was expressed and secreted in cell culture as described in Example 2. The isolated protein was then sequenced at its N-terminal region, yielding the N-terminal sequence shown in Fig. 8. This sequence, which is identified herein as SEQ ID NO:22, has the same N-terminal residues as native mature α_1 -antitrypsin.

Production of mature ATIII: In a preferred embodiment, the culture medium contains a MES buffer, pH 6.8. Western blot analysis of the ATIII-protein produced, shown in lanes 4 and 6 in Fig. 9, shows a band corresponding to ATIII (lane 1) in cell lines 42 and 46, when grown in the absence (but not in the presence) of sucrose.

Production of mature BPN': In one embodiment of the invention, in which BPN' is secreted as the proBPN' form of the enzyme, the chaperon "pro" moiety of the enzyme facilitates enzyme folding and is cleaved from the enzyme, leaving the active mature form of BPN'. In another embodiment, the mature enzyme is co-expressed and co-secreted with the "pro" chaperon moiety, with conversion of the enzyme to active form occurring in presence of the free chaperon (Eder *et al.*, *Biochem.* (1993) 32:18-26; Eder *et al.*, (1993) *J. Mol. Biol.* 223:293-304). In yet another embodiment of the invention, the BPN' is secreted in inactive form at a pH that may be in the 6-8 range, with subsequent activation of the inactive form, *e.g.*, after enzyme isolation, by exposure to the "pro" chaperon moiety, *e.g.*, immobilized to a solid support.

In both of these embodiments, the culture medium is maintained at a pH of between 5 and 6, preferably about 5.5 during the period of active expression and secretion of BPN', to keep the BPN', which is normally active at alkaline pH, at a pH below optimal activity.

Codon optimization to the host plant's most frequent codons yielded a severalfold enhancement in the level of expressed heterologous protein in cell culture as shown in Fig. 11. The extent of enhancement is seen from the Western blot analysis shown in Fig. 10 for two cells lines and further substantiated in Fig. 11. Lane 2 (second from left) in Fig. 10 shows a Western blot of BPN' obtained in culture from cells transformed with a native proBPN' coding sequence. Two bands observed correspond to a lower molecular weight protein whose approximately 35 kdal molecular weight corresponds to that of proBPN'. The upper band corresponds to a somewhat higher molecular weight species, possibly glycosylated.

The first lane in the figure shows BPN' polypeptides produced in culture by plant cells transformed with the codon-optimized proBPN' sequence identified by SEQ ID NO:21. For

comparative purposes, the same volume of culture medium, adjusted for cell density, was applied in both lanes 1 and 2. As seen, the amount of BPN' enzyme produced with a codon-optimized sequence was severalfold higher than for subtilisin BPN' produced with the native coding sequence. Further, a dark band or bands corresponding to mature peptide (molecular weight 27.5 kdal) was observed. However, it should be noted that directly above the band at 35kD is a more pronounced band which may be pro mature product yet to be cleaved into active form.

Fig. 11 compares the specific activity of BPN' codon-optimized (AP106) versus BPN' native (AP101) expression in rice callus cell culture, assayed using the chromogenic peptide substrate suc-Ala-Ala-Pro-Phe-pNA as described by DelMar, E.G. *et al.* (1979; Anal. Biochem. 99:316-320). As shown if Fig. 11, several of the cell lines transformed with codon-optimized chimeric genes produced levels of BPN', as evidenced by measured specific activity in culture medium, that were 2-5 times the highest levels observed for plant cells transformed with native proBPN' sequence.

In accordance with another aspect of the invention, it has been found that the transformed plant cell culture is able to express and secrete BPN' at a cell culture pH, pH 5.5, which largely inhibits self-degradation of mature, active BPN'. To assay for optimal pH conditions, the assay disclosed in DelMar, *et al.* (*supra*) is used to test the media derived from BPN' transformed cell lines under various pH conditions. Transformed rice callus cells are cultured in a MES medium under similar conditions as disclosed in Example 2, but where the pH of the medium is maintained at a selected pH between 5 and 8.0. At each pH, the total amount of expressed and secreted BPN' is determined by Western blot analysis. BPN' activity can be tested in the assay described by DelMar (*supra*).

V. Production of Mature Heterologous Protein in Germinating Seeds

In this embodiment, monocot cells transformed as above are used to regenerate plants, seeds from the plants are harvested and then germinated, and the mature protein is isolated from the germinated seeds.

Plant regeneration from cultured protoplasts or callus tissue is carried by standard methods, e.g., as described in Evans *et al.*, HANDBOOK OF PLANT CELL CULTURES Vol. 1: (MacMillan Publishing Co. New York, 1983); and Vasil I.R. (ed.), CELL CULTURE AND SOMATIC CELL GENETICS OF PLANTS, Acad. Press, Orlando, Vol. I, 1984, and Vol. III, 1986, and as described in the above-cited PCT application.

A. Seed Germination Conditions

The transgenic seeds obtained from the regenerated plants are harvested, and prepared for germination by an initial steeping step, in which the seeds immersed in or sprayed with water to

increase the moisture content of the seed to between 35-45%. This initiates germination. Steeping typically takes place in a steep tank which is typically fitted with a conical end to allow the seed to flow freely out. The addition of compressed air to oxygenate the steeping process is an option. The temperature is controlled at approximately 22°C depending on the seed.

5 After steeping, the seeds are transferred to a germination compartment which contains air saturated with water and is under controlled temperature and air flows. The typical temperatures are between 12-25°C and germination is permitted to continue for from 3 to 7 days.

10 Where the heterologous protein coding gene is operably linked to a inducible promoter requiring a metabolite such as sugar or plant hormone, e.g., 2 to 100 µM gibberellic acid, this metabolite is added, removed or depleted from the steeping water medium and/or is added to the water saturated air used during germination. The seed absorbs the aqueous medium and begins to germinate, expressing the heterologous protein. The medium may then be withdrawn and the malting begun, by maintaining the seeds in a moist temperature controlled aerated environment. In this way, the seeds may begin growth prior to expression, so that the expressed product is less 15 likely to be partially degraded or denatured during the process.

More specifically, the temperature during the imbibition or steeping phase will be maintained in the range of about 15-25°C, while the temperature during the germination will usually be about 20°C. The time for the imbibition will usually be from about 1 to 4 days, while the germination time will usually be an additional 1 to 10 days, more usually 3 to 7 days. Usually, the 20 time for the malting does not exceed about ten days. The period for the malting can be reduced by using plant hormones during the imbibition, particularly gibberellic acid.

To achieve maximum production of recombinant protein from malting, the malting procedure may be modified to accommodate de-hulled and de-embryonated seeds, as described in above-cited PCT application WO 95/14099. In the absence of sugars from the endosperm, there is 25 expected to be a 5 to 10 fold increase in RAmy3D promoter activity and thus expression of heterologous protein. Alternatively when embryoless half-seeds are incubated in 10 mM CaCl₂ and 5 µM gibberellic acid, there is a 50 fold increase in RAmy1A promoter activity.

Production of mature HSA: Following the germination conditions as outlined above and further detailed in Example 3, supernatant was analyzed by Western blot. Western blot analysis 30 shows production of HSA in germinating rice seeds, with seed samples taken 24, 72, and 120 hours after induction with gibberellin. HSA production was highest approximately 24 hours post-induction (lanes 3 and 4, Fig. 12). Bilirubin binding, a measure of correct folding of plant-produced HSA, is assayed according to the method presented in Example 3.

35 VI. Production of Mature Heterologous Protein in Maturing Seeds

In this embodiment, monocot cells transformed as above are used to regenerate plants, and seeds from the plants are allowed to mature, typically in the field, with consequent production of heterologous protein in the seeds.

Following seed maturation, the seeds and their heterologous proteins may be used directly, 5 that is, without protein isolation, where for example, the heterologous protein is intended to confer a benefit on the seed as a whole, for example, to enrich the seed in the selected protein.

Alternatively, the seeds may be fractionated by standard methods to obtain the heterologous protein in enriched or purified form. In one general approach, the seed is first milled, then suspended in a suitable extraction medium, e.g., an aqueous or an organic solvent, to extract the 10 protein or metabolite of interest. If desired the heterologous protein can be further fractionated and purified, using standard purification methods.

The following examples are provided by way of illustration only and not by way of limitation. Those of skill will readily recognize a variety of noncritical parameters which could be 15 changed or modified to yield essentially similar results.

General Methods

Generally, the nomenclature and laboratory procedures with respect to standard recombinant DNA technology can be found in Sambrook, et al., MOLECULAR CLONING - A LABORATORY 20 MANUAL, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 1989 and in S.B. Gelvin and R.A. Schilperoort, PLANT MOLECULAR BIOLOGY, 1988. Other general references are provided throughout this document. The procedures therein are known in the art and are provided for the convenience of the reader.

25

Example 1

Construction of a Transforming Vector Containing a Codon-Optimized α_1 -antitrypsin Sequence

A. Hygromycin Resistance Gene Insertion:

The 3 kb *Bam*HI fragment containing the 35S promoter-Hph-NOS was removed from the 30 plasmid pMON410 (Monsanto, St. Louis, MO) and placed into an site-directed mutagenized *Bgl*II site in the pUC18 at 1463 to form the plasmid pUCH18+.

B. Terminator Insertion:

pOSg1ABK5 is a 5 kb *Bam*HI-*Kpn*I fragment from lambda clone λ OSg1A (Huang, N., et 35 al., (1990) Nuc. Acids Res. 18:7007) cloned into pBluescript KS- (Stratagene, San Diego, CA).

Plasmid pOSg1ABK5 was digested with *Msp*I and blunted with T4 DNA polymerase followed by *Spe*I digestion. The 350 bp terminator fragment was subcloned into pUC19 (New England BioLabs, Beverly, MA), which had been digested with *Bam*HI, blunted with T4 DNA polymerase and digested with *Xba*I, to form pUC19/terminator.

5

C. RAmy3D Promoter Insertion:

A 1.1 kb *Nhe*I-*Pst*I fragment derived from p1AS1.5 (Huang, N. et al. (1993) Plant Mol. Biol. 23:737-747), was cloned into the vector pGEM5zf- [multiple cloning site (MCS) (Promega, Madison, WI): *Apal*, *Aat*II, *Sph*I, *Nco*I, *Sst*II, *Eco*RV, *Spe*I, *Not*I, *Pst*I, *Sal*I, *Nde*I, *Sac*I, *Mlu*I, *Nsi*I] at the *Spe*I and *Pst*I sites to form pGEM5zf-(3D/*Nhe*I-*Pst*I). pGEM5zf-(3D/*Nhe*I-*Pst*I) was then digested with *Pst*I and *Sac*I, and two non-kinased 30mers having the complementary sequences 5' GCTTG ACCTG TAACT CGGGC CAGGC GAGCT 3' (SEQ ID NO:23) and 5' CGCCT AGCCC GAGTT ACAGG TCAAG CAGCT 3' (SEQ ID NO:24) were ligated in to form p3DProSig. The promoter fragment prepared by digesting p3DProSig with *Nco*I, blunting with T4 DNA polymerase, and digesting with *Sst*I was subcloned into pUC19/terminator which had been digested with *Eco*RI, blunted with T4 DNA polymerase and digested with *Sst*I, to form p3DProSigEND.

D. Multiple Cloning Site Insertion:

20 p3DProSigEND was digested with *Sst*I and *Sma*I followed by the ligation of a new synthetic linker fragment constructed with the non-kinased complementary oligonucleotides 5' AGCTC CATGG CCGTG GCTCG AGTCT AGACG CGTCC CC 3' (SEQ ID NO:25) and 5' GGGGA CGCGT CTAGA CTCGA GCCAC GGCCA TGG 3' (SEQ ID NO:26) to form p3DProSigENDlink.

25

E. p3DProSigENDlink Flanking Site Modification:

p3DProSigENDlink was digested with *Sal*I and blunted with T4 DNA polymerase followed by *Eco*RV digestion. The blunt fragment was then inserted into pBluescript KS+ (Stratagene) in the *Eco*RV site so that the *Hind*III site is proximal to the promoter and the *Eco*RI is proximal to the 30 terminator sequence. The *Hind*III-*Eco*RI fragment was then moved into the polylinker of pUCH18+ to form the p3Dv1.0 expression vector.

F. RAmy1A Promoter Insertion:

A 1.9 kb *Nhe*I-*Pst*I fragment derived from subclone pOSG2CA2.3 from lambda clone λ OSg2 (Huang et al. (1990) Plant Mol. Biol. 14:655-668), was cloned into the vector pGEM5zf- at

the *Spe*I and *Pst*I sites to form pGEM5zf-(1A/*Nhe*I-*Pst*I). pGEM5zf-(1A/*Nhe*I-*Pst*I) was digested with *Pst*I and *Sac*I and two non-kinased 35mers and four kinased 32mers were ligated in, with the complementary sequences as follows: 5' GCATG CAGGT GCTGA ACACC ATGGT GAACA AACAC 3' (SEQ ID NO:27); 5' TTCTT GTCCC TTTCG GTCCT CATCG TCCTC CT 3' (SEQ ID NO:28); 5' TGGCC TCTCC TCCAA CTTGA CAGCC GGGAG CT 3' (SEQ ID O:29); 5' TTCAC CATGG TGTTC AGCAC CTGCA TGCTG CA 3' (SEQ ID NO:30); 5' CGATG AGGAC CGAAA GGGAC AAGAA GTGTT TG 3' (SEQ ID NO:31); 5' CCCGG CTGTC AAGTT GGAGG AGAGG CCAAG GAGGA 3' (SEQ ID NO:32) to form p1AProSig. The *Hind*III-*Sac*I 0.8 kb promoter fragment was subcloned from p1AProSig into the p3Dv1.0 vector digested with *Hind*III-*Sac*I to yield the p1Av1.0 expression vector.

G. Construction of p3D-AAT Plasmid

Two PCR primers were used to amplify a fragment encoding AAT according to the sequence disclosed as Genbank Accession No. K01396: N-terminal primer 5' GAGGA TCCCC AGGGA GATGC TGCCC AGAA 3' (SEQ ID NO:33) and C-terminal primer 5' CGCGC TCGAG TTATT TTTGG GTGGG ATTCA CCAC 3' (SEQ ID NO:34). The N-terminal primer amplifies to a blunt site for in-frame insertion with the end of the p3D signal peptide and the C-terminal primer contains a *Xho*I site for cloning the fragment into the vector as shown in Figs. 3A and 3B. Alternatively, the sequence encoding mature AAT (SEQ ID NO:8) or codon-optimized AAT may be chemically synthesized using techniques known in the art, incorporating a *Xho*I restriction site 3' of the termination codon for insertion into the expression vector as described above.

Example 2

Production of mature α -antitrypsin in cell culture

After selection of transgenic callus, callus cells were suspended in liquid culture containing AA2 media (Thompson, J.A., et al., *Plant Science* 47:123 (1986), at 3% sucrose, pH 5.8. Thereafter, the cells were shifted to phosphate-buffered media (20 mM phosphate buffer, pH 6.8) using 10 mL multi-well tissue culture plates and shaken at 120 rpm in the dark for 48 hours. The supernatant was then removed and stored at -80°C prior to western blot analysis.

Supernatants were concentrated using Centricon-10 filters (Amicon cat. #4207) and washed with induction media to remove substances interfering with electrophoretic migration. Samples were concentrated approximately 10 fold, and mature AAT was purified by SDS PAGE electrophoresis. The purified protein was extracted from the electrophoresis medium, and sequenced at its N-terminus, giving the sequence shown in Fig. 8, identified herein as SEQ ID NO:22.

Example 3

HSA Induction in Germinating Seeds

5 After selection of transgenic plants which tested positive for the presence of a codon-optimized HSA gene driven by the GA₃-responsive RAmy1A promoter, seeds were harvested and imbibed for 24 hours with 100 rpm orbital shaking in the dark at 25°C. GA₃ was added to a final concentration of 5μM and incubated for an additional 24-120 hours. Total soluble protein was isolated by double grinding each seed in 120 μl grinding buffer and centrifuging at 23,000 x g for 1
10 minute at 4°C. The clear supernatant was carefully removed from the pellet and transferred to a fresh tube.

Bilirubin binding assay

15 Bilirubin binding to its high-affinity site on mature HSA is assayed using the method described by Jacobsen, J. *et al.* (1974; Clin. Chem. 20:783) and Reed, R.G. *et al.* (1975; Biochemistry 14:4578-4583). Briefly, the concentration of free bilirubin in equilibrium with protein-bound bilirubin is determined by the rate of peroxide-peroxidase catalyzed oxidation of free bilirubin. Stock solutions of bilirubin (Nutritional Biochemicals Corp.) are prepared fresh daily in 5 mM NaOH containing 1mM EDTA and the concentration determined using a molar absorptivity of 47,500 M⁻¹ cm⁻¹ at 440 nm. An aliquot containing between 5 and 30 nmol bilirubin is added to a
20 1 cm cuvette containing 1 ml PBS and approximately 30 nmol HSA at 37°C. An absorbance spectrum between 500 and 350 nm is recorded. Aliquots of horseradish peroxidase (Sigma), 0.05 mg/ml in PBS, and 0.05% ethyl hydrogen peroxide (Ferrosan; Malmö Sweden) are added and the change in absorbance at λ_{max} is recorded for 3-5 minutes. The concentrations of free and bound
25 bilirubin calculated from the oxidation rate observed using varying concentrations of total bilirubin are used to construct a Scatchard plot from which the association constant for a single binding site is determined.

Although the invention has been described with reference to particular embodiments, it will be appreciated that a variety of changes and modifications can be made without departing from the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: Applied Phytologics, Inc.

(ii) TITLE OF THE INVENTION: Production of Mature Proteins in Plants

(iii) NUMBER OF SEQUENCES: 34

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Dehlinger & Associates

(B) STREET: P.O. Box 60850

(C) CITY: Palo Alto

(D) STATE: CA

(E) COUNTRY: USA

(F) ZIP: 94306

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette

(B) COMPUTER: IBM Compatible

(C) OPERATING SYSTEM: DOS

(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: PCT/US98/03068

(B) FILING DATE: 13-FEB-1998

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 60/038,169

(B) FILING DATE: 13-FEB-1997

(A) APPLICATION NUMBER: 60/037,991

(B) FILING DATE: 13-FEB-1997

(A) APPLICATION NUMBER: 60/038,170

(B) FILING DATE: 13-FEB-1997

(A) APPLICATION NUMBER: 60/038,168

(B) FILING DATE: 13-FEB-1997

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Petithory, Joanne R

(B) REGISTRATION NUMBER: P42,995

(C) REFERENCE/DOCKET NUMBER: 0665-0007.41

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 650-324-0880

(B) TELEFAX: 650-324-0960

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:

(B) CLONE: 3D signal peptide sequence

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Lys Asn Thr Ser Ser Leu Cys Leu Leu Leu Val Val Leu Cys
1 5 10 15
Ser Leu Thr Cys Asn Ser Gly Gln Ala
20 25

5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 75 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:

- (B) CLONE: native 3D signal peptide DNA sequence

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATGAAGAACAA CCAGCAGCTT GTGTTGCTG CTCCCTCGTGG TGCTCTGCAG CTTGACCTGT
20 AACTCGGGCC AGGCG 60
75

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 75 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:

- (B) CLONE: codon-optimized 3D signal peptide DNA sequence

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATGAAGAACAA CCTCCTCCCT CTGCCTCCTG CTGCTCGTGG TCCTCTGCTC CCTGACCTGC
35 AACAGCGGCC AGGCC 60
75

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

- (vii) IMMEDIATE SOURCE:
(B) CLONE: RAmy1A signal peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Val Asn Lys His Phe Leu Ser Leu Ser Val Leu Ile Val Leu Leu
50 1 5 10 15
Gly Leu Ser Ser Asn Leu Thr Ala Gly
20 25

55

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 51 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:

- (B) CLONE: RAmy 1A 5' untranslated region (UTR)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

(2) INFORMATION FOR SEQ ID NO:6:

5

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 321 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

10

- (vii) IMMEDIATE SOURCE:
 (B) CLONE: RAmy 1A 3' untranslated region (UTR)

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCGCACGGATG ACGAGACTCT CAGTTAGCA GATTAAACCT GCGATTTTTA CCCTGACC GG	60
TATACGTATA TACGTGCCGG CAACGAGCTG TATCCGATCC GAATTACGGA TGCAATTGTC	120
CACGAAGTAC TTCCTCCGTA AATAAAGTAG GATCAGGGAC ATACATTGT ATGGTTTAC	180
GAATAATGCT ATGCAATAAA ATTTGCACTG CTTAATGCTT ATGCATTTT GCTTGGTTCG	240
ATTGTACTGG TGAATTATTG TTACTGTTCT TTTTACTTCT CGAGTGGCAG TATTGTTCTT	300
CTACGAAAAT TTGATGCGTA G	321

20

(2) INFORMATION FOR SEQ ID NO:7:

25

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 394 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

30

- (ii) MOLECULE TYPE: protein
 (vii) IMMEDIATE SOURCE:
 (B) CLONE: mature AAT amino acid sequence

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Glu Asp Pro Gln Gly Asp Ala Ala Gln Lys Thr Asp Thr Ser His His	
1 5 10 15	
Asp Gln Asp His Pro Thr Phe Asn Lys Ile Thr Pro Asn Leu Ala Glu	
20 25 30	
Phe Ala Phe Ser Leu Tyr Arg Gln Leu Ala His Gln Ser Asn Ser Thr	
35 40 45	
Asn Ile Phe Phe Ser Pro Val Ser Ile Ala Thr Ala Phe Ala Met Leu	
50 55 60	
Ser Leu Gly Thr Lys Ala Asp Thr His Asp Glu Ile Leu Glu Gly Leu	
65 70 75 80	
Asn Phe Asn Leu Thr Glu Ile Pro Glu Ala Gln Ile His Glu Gly Phe	
85 90 95	
Gln Glu Leu Leu Arg Thr Leu Asn Gln Pro Asp Ser Gln Leu Gln Leu	
100 105 110	
Thr Thr Gly Asn Gly Leu Phe Leu Ser Glu Gly Leu Lys Leu Val Asp	
115 120 125	
Lys Phe Leu Glu Asp Val Lys Lys Leu Tyr His Ser Glu Ala Phe Thr	
130 135 140	
Val Asn Phe Gly Asp Thr Glu Glu Ala Lys Lys Gln Ile Asn Asp Tyr	
145 150 155 160	
Val Glu Lys Gly Thr Gln Gly Lys Ile Val Asp Leu Val Lys Glu Leu	
165 170 175	
Asp Arg Asp Thr Val Phe Ala Leu Val Asn Tyr Ile Phe Phe Lys Gly	
180 185 190	
Lys Trp Glu Arg Pro Phe Glu Val Lys Asp Thr Glu Glu Asp Phe	
195 200 205	
His Val Asp Gln Val Thr Thr Val Lys Val Pro Met Met Lys Arg Leu	
210 215 220	
Gly Met Phe Asn Ile Gln His Cys Lys Lys Leu Ser Ser Trp Val Leu	
225 230 235 240	
Leu Met Lys Tyr Leu Gly Asn Ala Thr Ala Ile Phe Phe Leu Pro Asp	
245 250 255	

Glu	Gly	Lys	Leu	Gln	His	Leu	Glu	Asn	Glu	Leu	Thr	His	Asp	Ile	Ile
			260				265	.				270			
Thr	Lys	Phe	Leu	Glu	Asn	Glu	Asp	Arg	Arg	Ser	Ala	Ser	Leu	His	Leu
			275				280				285				
Pro	Lys	Leu	Ser	Ile	Thr	Gly	Thr	Tyr	Asp	Leu	Lys	Ser	Val	Leu	Gly
			290			295				300					
Gln	Leu	Gly	Ile	Thr	Lys	Val	Phe	Ser	Asn	Gly	Ala	Asp	Leu	Ser	Gly
			305			310				315					320
Val	Thr	Glu	Glu	Ala	Pro	Leu	Lys	Leu	Ser	Lys	Ala	Val	His	Lys	Ala
					325				330						335
Val	Leu	Thr	Ile	Asp	Glu	Lys	Gly	Thr	Glu	Ala	Ala	Gly	Ala	Met	Phe
					340				345						350
Leu	Glu	Ala	Ile	Pro	Met	Ser	Ile	Pro	Pro	Glu	Val	Lys	Phe	Asn	Lys
					355				360			365			
Pro	Phe	Val	Phe	Leu	Met	Ile	Glu	Gln	Asn	Thr	Lys	Ser	Pro	Leu	Phe
					370			375			380				
Met	Gly	Lys	Val	Val	Asn	Pro	Thr	Gln	Lys						
					385			390							

20 (2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:

 - (A) LENGTH: 1185 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: native coding sequence of mature AAT

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GAGGATCCCC	AGGGAGATGC	TGCCCAAG	ACAGATACT	CCCACCATGA	TCAGGATCAC	60
CCAACCTTCA	ACAAGATCAC	CCCCAACCTG	GCTGAGTTCG	CCTTCAGCCT	ATACCGCCAG	120
CTGGCACACC	AGTCCAACAG	CACCAATATC	TTCTTCTCCC	CAGTGAGCAT	CGCTACAGCC	180
TTTGCAATGC	TCTCCCTGGG	GACCAAGGCT	GACACTCAGC	ATGAAATCCT	GGAGGGCCTG	240
AATTTCACCC	TCACGGAGAT	TCCGGAGGCT	CAGATCCATG	AAGGCTTCCA	GGAACTCCTC	300
CGTACCCCTCA	ACCAGCCAGA	CAGCCAGCTC	CAGCTGACCA	CCGGCAATGG	CCTGTTCCCTC	360
AGCGAGGGCC	TGAAGCTAGT	GGATAAGTT	TTGGAGGATG	TTAAAAGTT	GTACCACTCA	420
GAAGCCTTCA	CTGTCAACTT	CGGGGACACC	GAAGAGGCCA	AGAAACAGAT	CAACGATTAC	480
GTGGAGAAGG	GTACTCAAGG	GAAAATGTG	GATTTGGTCA	AGGAGCTTGA	CAGAGACACA	540
GTTTTGCTC	TGGTGAATT	CATCTTCTT	AAAGGCAAAT	GGGAGAGACC	CTTGAAAGTC	600
AAGGACACCG	AGGAAGAGGA	CTTCCACGTG	GACCAGGTGA	CCACCGTGA	GGTGCCTATG	660
ATGAAGCGTT	TAGGCATGTT	TAACATCCAG	CACTGTAAGA	AGCTGTCCAG	CTGGGTGCTG	720
CTGATGAAAT	ACCTGGGCAA	TGCCACCGC	ATCTTCTTCC	TGCCTGATGA	GGGGAAACTA	780
CAGCACCTGG	AAAATGAACT	CACCCACGAT	ATCATCACCA	AGTCTCTGGA	AAATGAAGAC	840
AGAAGGCTG	CCAGCTTACA	TTTACCCAAA	CTGTCCTTAA	CTGGAACCTA	TGATCTGAAG	900
AGCCTCTG	GTCAACTGGG	CATCACTAAG	GTCTTCAGCA	ATGGGGCTGA	CCTCTCCGGG	960
GTCACAGAGG	AGGCACCCCT	GAAGCTCTCC	AAGGCCGTGC	ATAAGGCTGT	GCTGACCATC	1020
GACGAGAAAG	GGACTGAAGC	TGCTGGGGCC	ATGTTTTAG	AGGCCATACC	CATGTCTATC	1080
CCCCCGAGG	TCAAGTTCAA	CAAACCTTT	GTCTTCTTAA	TGATTGAACA	AAATACCAAG	1140
TCTCCCCCTCT	TCATGGGAAA	AGTGGTGAAT	CCCACCCAAA	AATAA		1185

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:

 - (A) LENGTH: 432 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(vii) IMMEDIATE SOURCE:

(B) CLONE: mature ATIII aa sequence

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

His Gly Ser Pro Val Asp Ile Cys Thr Ala Lys Pro Arg Asp Ile Pro

	1	5	10	15
	Met Asn Pro Met Cys Ile Tyr Arg Ser Pro Glu Lys Lys Ala Thr Glu			
	20	25	30	
5	Asp Glu Gly Ser Glu Gln Lys Ile Pro Glu Ala Thr Asn Arg Arg Val			
	35	40	45	
	Trp Glu Leu Ser Lys Ala Asn Ser Arg Phe Ala Thr Thr Phe Tyr Gln			
	50	55	60	
10	His Leu Ala Asp Ser Lys Asn Asp Asn Asp Asn Ile Phe Leu Ser Pro			
	65	70	75	80
	Leu Ser Ile Ser Thr Ala Phe Ala Met Thr Lys Leu Gly Ala Cys Asn			
	85	90	95	
	Asp Thr Leu Gln Gln Leu Met Glu Val Phe Lys Phe Asp Thr Ile Ser			
	100	105	110	
15	Glu Lys Thr Ser Asp Gln Ile His Phe Phe Phe Ala Lys Leu Asn Cys			
	115	120	125	
	Arg Leu Tyr Arg Lys Ala Asn Lys Ser Ser Lys Leu Val Ser Ala Asn			
	130	135	140	
	Arg Leu Phe Gly Asp Lys Ser Leu Thr Phe Asn Glu Thr Tyr Gln Asp			
20	145	150	155	160
	Ile Ser Glu Leu Val Tyr Gly Ala Lys Leu Gln Pro Leu Asp Phe Lys			
	165	170	175	
	Glu Asn Ala Glu Gln Ser Arg Ala Ala Ile Asn Lys Trp Val Ser Asn			
	180	185	190	
25	Lys Thr Glu Gly Arg Ile Thr Asp Val Ile Pro Ser Glu Ala Ile Asn			
	195	200	205	
	Glu Leu Thr Val Leu Val Leu Val Asn Thr Ile Tyr Phe Lys Gly Leu			
	210	215	220	
30	Trp Lys Ser Lys Phe Ser Pro Glu Asn Thr Arg Lys Glu Leu Phe Tyr			
	225	230	235	240
	Lys Ala Asp Gly Glu Ser Cys Ser Ala Ser Met Met Tyr Gln Glu Gly			
	245	250	255	
	Lys Phe Arg Tyr Arg Arg Val Ala Glu Gly Thr Gln Val Leu Glu Leu			
	260	265	270	
35	Pro Phe Lys Gly Asp Asp Ile Thr Met Val Leu Ile Leu Pro Lys Pro			
	275	280	285	
	Glu Lys Ser Leu Ala Lys Val Glu Lys Glu Leu Thr Pro Glu Val Leu			
	290	295	300	
	Gln Glu Trp Leu Asp Glu Leu Glu Glu Met Met Leu Val Val His Met			
40	305	310	315	320
	Pro Arg Phe Arg Ile Glu Asp Gly Phe Ser Leu Lys Glu Gln Leu Gln			
	325	330	335	
	Asp Met Gly Leu Val Asp Leu Phe Ser Pro Glu Lys Ser Lys Leu Pro			
	340	345	350	
45	Gly Ile Val Ala Glu Gly Arg Asp Asp Leu Tyr Val Ser Asp Ala Phe			
	355	360	365	
	His Lys Ala Phe Leu Glu Val Asn Glu Glu Gly Ser Glu Ala Ala Ala			
	370	375	380	
	Ser Thr Ala Val Val Ile Ala Gly Arg Ser Leu Asn Pro Asn Arg Val			
50	385	390	395	400
	Thr Phe Lys Ala Asn Arg Pro Phe Leu Val Phe Ile Arg Glu Val Pro			
	405	410	415	
	Leu Asn Thr Ile Ile Phe Met Gly Arg Val Ala Asn Pro Cys Val Lys			
	420	425	430	

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1299 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(vi) IMMEDIATE SOURCE:

- (B) CLONE: native ATIII DNA sequence

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

	CACGGAAAGCC	CTGTGGACAT	CTGCACAGCC	AAGCCGCGGG	ACATTCCCAT	GAATCCCATG	60
	TGCATTTACC	GCTCCCCGGA	GAAGAAGGCA	ACTGAGGATG	AGGGCTCAGA	ACAGAAGATC	120
	CCGGAGGCCA	CCAACCGGGC	TGTCTGGAA	CTGTCCAAGG	CCAATCCCC	CTTGCTACC	180
5	ACTTTCTATC	AGCACCTGGC	AGATCCAAG	AATGACAATG	ATAACATTT	CCTGTCACCC	240
	CTGAGTATCT	CCACGGCTT	TGCTATGACC	AAGCTGGGTG	CCTGTAAATGA	CACCCCTCCAG	300
	CAACTGATGG	AGGTATTTAA	GTTTGACACC	ATATCTGAGA	AAACATCTGA	TCAGATCCAC	360
	TTCTTCTTG	CCAAACTGAA	CTGCCACTC	TATCGAAAAG	CCAACAAATC	CTCCAAGTTA	420
	GTATCAGCCA	ATCGCCTTT	TGGAGACAA	TCCCTTACCT	TCAATGAGAC	CTACCAGGAC	480
10	ATCAGTGAGT	TGGTATATGG	AGCCAAGCTC	CAGCCCCCTGG	ACTTCAAGGA	AAATGCAGAG	540
	CAATCCAGAG	CGGCCATCAA	CAAATGGGTG	TCCAATAAGA	CCGAAGGCCG	AATCACCGAT	600
	GTCATTCCT	CGGAAGCCAT	CAATGAGCTC	ACTGTTCTGG	TGCTGGTTAA	CACCATTAC	660
	TTCAAGGGCC	TGTGGAAGTC	AAAGTTCAGC	CCTGAGAAACA	CAAGGAAGGA	ACTGTTCTAC	720
15	AAGGCTGATG	GAGAGTCGTG	TTCAGCATCT	ATGATGTACC	AGGAAGGCAA	GTTCGTTAT	780
	CGGCGGTGG	CTGAAGGCAC	CCAGGTGCTT	GAGTTGCCCT	TCAAAGGTGA	TGACATCACC	840
	ATGGTCTCA	TCTTGCCCAA	GCCTGAGAAG	AGCCTGGCCA	AGGTGGAGAA	GGAACTCACC	900
	CCAGAGGTGC	TGCAGGAGTG	GCTGGATGAA	TTGGAGGAGA	TGATGCTGGT	GGTTCACATG	960
	CCCCGTTCC	GCATTGAGGA	CGGCTTCAGT	TTGAAGGAGC	AGCTGCAAGA	CATGGGCCTT	1020
	GTCGATCTGT	TCAGCCCTGA	AAAGTCCAAA	CTCCCAGGT	TTGTTGCAGA	AGGCGAGAT	1080
20	GACCTCTATG	TCTCAGATGC	ATTCCATAAG	GCATTTCCTG	AGGTAAATGA	AGAAGGCAGT	1140
	GAAGCAGCTG	CAAGTACCGC	TGTTGTGATT	GCTGGCGTT	CGCTAAACCC	CAACAGGGTG	1200
	ACTTTCAAGG	CCAACAGGCC	CTTCTGGTT	TTTATAAGAG	AAGTCCCTCT	GAACACTATT	1260
	ATCTTCATGG	GCAGAGTAGC	CAACCCTTGT	GTAAAGTAA			1299

(2) INFORMATION FOR SEQ ID NO:11:

- 25 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 585 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear
- 30 (ii) MOLECULE TYPE: protein
 (vii) IMMEDIATE SOURCE:
 (B) CLONE: mature HSA amino acid sequence

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

	Asp	Ala	His	Lys	Ser	Glu	Val	Ala	His	Arg	Phe	Lys	Asp	Leu	Gly	Glu
	1								5			10			15	
	Glu	Asn	Phe	Lys	Ala	Leu	Val	Leu	Ile	Ala	Phe	Ala	Gln	Tyr	Leu	Gln
									20			25			30	
40	Gln	Cys	Pro	Phe	Glu	Asp	His	Val	Lys	Leu	Val	Asn	Glu	Val	Thr	Glu
									35			40			45	
	Phe	Ala	Lys	Thr	Cys	Val	Ala	Asp	Glu	Ser	Ala	Glu	Asn	Cys	Asp	Lys
									50			55			60	
45	Ser	Leu	His	Thr	Leu	Phe	Gly	Asp	Lys	Leu	Cys	Thr	Val	Ala	Thr	Leu
									65			70			75	
	Arg	Glu	Thr	Tyr	Gly	Glu	Met	Ala	Asp	Cys	Cys	Ala	Lys	Gln	Glu	Pro
									85			90			95	
50	Glu	Arg	Asn	Glu	Cys	Phe	Leu	Gln	His	Lys	Asp	Asp	Asn	Pro	Asn	Leu
									100			105			110	
	Pro	Arg	Leu	Val	Arg	Pro	Glu	Val	Asp	Val	Met	Cys	Thr	Ala	Phe	His
									115			120			125	
	Asp	Asn	Glu	Glu	Thr	Phe	Leu	Lys	Lys	Tyr	Leu	Tyr	Glu	Ile	Ala	Arg
									130			135			140	
55	Arg	His	Pro	Tyr	Phe	Tyr	Ala	Pro	Glu	Leu	Leu	Phe	Phe	Ala	Lys	Arg
									145			150			155	
	Tyr	Lys	Ala	Ala	Phe	Thr	Glu	Cys	Cys	Gln	Ala	Ala	Asp	Lys	Ala	Ala
									165			170			175	
60	Cys	Leu	Leu	Pro	Lys	Leu	Asp	Glu	Leu	Arg	Asp	Glu	Gly	Lys	Ala	Ser
									180			185			190	
	Ser	Ala	Lys	Gln	Arg	Leu	Lys	Cys	Ala	Ser	Leu	Gln	Lys	Phe	Gly	Glu
									195			200			205	
65	Arg	Ala	Phe	Lys	Ala	Trp	Ala	Val	Ala	Arg	Leu	Ser	Gln	Arg	Phe	Pro
									210			215			220	
	Lys	Ala	Glu	Phe	Ala	Glu	Val	Ser	Lys	Leu	Val	Thr	Asp	Leu	Thr	Lys
									225			230			235	
	Val	His	Thr	Glu	Cys	Cys	His	Gly	Asp	Leu	Leu	Glu	Cys	Ala	Asp	Asp

	245	250	255
	Arg Ala Asp Leu Ala Lys Tyr Ile Cys Glu Asn Gln Asp Ser Ile Ser		
	260	265	270
5	Ser Lys Leu Lys Glu Cys Cys Glu Lys Pro Leu Leu Glu Lys Ser His		
	275	280	285
	Cys Ile Ala Glu Val Glu Asn Asp Glu Met Pro Ala Asp Leu Pro Ser		
	290	295	300
	Leu Ala Ala Asp Phe Val Glu Ser Lys Asp Val Cys Lys Asn Tyr Ala		
	305	310	315
10	Glu Ala Lys Asp Val Phe Leu Gly Met Phe Leu Tyr Glu Tyr Ala Arg		
	325	330	335
	Arg His Pro Asp Tyr Ser Val Val Leu Leu Leu Arg Leu Ala Lys Thr		
	340	345	350
	Tyr Glu Thr Thr Leu Glu Lys Cys Cys Ala Ala Asp Pro His Glu		
15	355	360	365
	Cys Tyr Ala Lys Val Phe Asp Glu Phe Lys Pro Leu Val Glu Glu Pro		
	370	375	380
	Gln Asn Leu Ile Lys Gln Asn Cys Glu Leu Phe Lys Gln Leu Gly Glu		
	385	390	395
20	Tyr Lys Phe Gln Asn Ala Leu Leu Val Arg Tyr Thr Lys Lys Val Pro		
	405	410	415
	Gln Val Ser Thr Pro Thr Leu Val Glu Val Ser Arg Asn Leu Gly Lys		
	420	425	430
	Val Gly Ser Lys Cys Cys Lys His Pro Glu Ala Lys Arg Met Pro Cys		
25	435	440	445
	Ala Glu Asp Tyr Leu Ser Val Val Leu Asn Gln Leu Cys Val Leu His		
	450	455	460
	Glu Lys Thr Pro Val Ser Asp Arg Val Thr Lys Cys Cys Thr Glu Ser		
	465	470	475
30	480	485	490
	Leu Val Asn Arg Arg Pro Cys Phe Ser Ala Leu Glu Val Asp Glu Thr		
	495		
	Tyr Val Pro Lys Glu Phe Asn Ala Glu Thr Phe Thr Phe His Ala Asp		
	500	505	510
	Ile Cys Thr Leu Ser Glu Lys Glu Arg Gln Ile Lys Lys Gln Thr Ala		
35	515	520	525
	Leu Val Glu Leu Val Lys His Lys Pro Lys Ala Thr Lys Glu Gln Leu		
	530	535	540
	Lys Ala Val Met Asp Asp Phe Ala Ala Phe Val Glu Lys Cys Cys Lys		
	545	550	555
40	560		
	Ala Asp Asp Lys Glu Thr Cys Phe Ala Glu Glu Gly Lys Lys Leu Val		
	565	570	575
	Ala Ala Ser Gln Ala Ala Leu Gly Leu		
	580	585	

45 (2) INFORMATION FOR SEQ ID NO:12:

	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 1865 base pairs	
50	(B) TYPE: nucleic acid	
	(C) STRANDEDNESS: single	
	(D) TOPOLOGY: linear	
	(vii) IMMEDIATE SOURCE:	
	(B) CLONE: native coding sequence of mature HSA	
55	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:	
	AGATGCACAC AAGAGTGAGG TTGCTCATCG GTTTAAAGAT TTGGGAGAAG AAAATTCAA	60
60	AGCCTTGGTG TTGATTGCT TTGCTCAGTA TCTTCAGCAG TGTCATTTG AAGATCATGT	120
	AAAAATTAGTG AATGAAGTAA CTGAATTTCG AAAAACATGT GTAGCTGATG AGTCAGCTGA	180
	AAATTGTGAC AAATCACTTC ATACCCTTT TGGAGACAAA TTATGCACAG TTGCAACTCT	240
	TCGTGAAACC TATGGTGAAA TGGCTGACTG CTGTGAAAA CAAGAACCTG AGAGAAATGA	300
	ATGCTTCTTG CAACACAAAG ATGACAACCC AAACCTCCCC CGATTGGTGA GACCAGAGGT	360
65	TGATGTGATG TGCAC TGCTT TTCATGACAA TGAAGAGACA TTTTGAAAA AATACTTATA	420
	TGAAATTGCC AGAAGACATC CTTACTTTA TGCCCCGGAA CTCCTTTCT TTGCTAAAAG	480
	GTATAAAGCT GCTTTACAG AATGTTGCCA AGCTGCTGAT AAAGCTGCC GCCTGTTGCC	540
	AAAGCTCGAT GAACTTCGGG ATGAAGGGAA GGCTCGTCT GCCAAACAGA GACTCAAATG	600

	TGCCAGTCTC CAAAAATTG GAGAAAGAGC TTTCAAAGCA	TGGGCAGTGG CTCGCCCTGAG	660
	CCAGAGATTT CCCAAAGCTG AGTTGCAGA AGTTCCAAG	TTAGTGCACAG ATCTTACCAA	720
	AGTCCACACG GAATGCTGCC ATGGAGATCT GCTTGAATGT	GCTGATGACA GGGCGGACCT	780
5	TGCCAAGTAT ATCTGTGAAA ATCAGGATTC GATCTCCAGT	AAACTGAAGG AATGCTGTGA	840
	AAAACCTCTG TTGGAAAAAT CCCACTGCAT TGCCGAAGTG	GAAAATGATG AGATGCCCTGC	900
	TGACTTGCCT TCATTAGCTG CTGATTGTTG TGAAAGTAAG	GATGTTGCA AAAACTATGC	960
	TGAGGCAAAG GATGCTTCC TGGGCATGTT TTTGTATGAA	TATGCAAGAA GGCATCCTGA	1020
10	TTACTCTGTC GTGCTGCTGC TGAGACTTGC CAAGACATAT	GAAACCACTC TAGAGAAGTG	1080
	CTGTGCCGCT GCAGATCCTC ATGAATGCTA TGCCAAAGTG	TTCGATGAAT TAAACCTCT	1140
	TGTGGAAAGAG CCTCAGAATT TAATCAAACA AAACCTGTGAG	CTTTTTAAGC AGCTTGGAGA	1200
	GTACAAATTG CAGAATGCGC TATTAGTTCG TTACACCAAG AAAGTACCCC AAGTGTCAAC		1260
15	TCCAACCTT GTAGAGGTCT CAAGAAAACCT AGGAAAAGTG	GGCAGCAAT GTTGTAAACA	1320
	TCTCTGAAGCA AAAAGAATGC CCTGTGCAGA AGACTATCTA	TCCGTGGTCC TGAACCAAGTT	1380
	ATGTGTGTTG CATGAGAAAA CGCCAGTAAG TGACAGAGTC	ACAAAATGCT GCACAGAGTC	1440
	CTTGGTGAAC AGGGCACCAT GCTTTTCAGC TCTGGAAGTC	GATGAAACAT ACGTTCCCAA	1500
20	AGAGTTTAAT GCTGAAACAT TCACCTTCCA TGCGAGATATA	TGCACACTTT CTGAGAAGGA	1560
	GAGACAAATC AAGAACAAAA CTGCACTTGT TGAGCTTGTG	AAACACAAAGC CCAAGGCAAC	1620
	AAAAGAGCAA CTGAAAGCTG TTATGGATGA TTTCGCAGCT	TTTGTAGAGA AGTGCCTGCAA	1680
	GGCTGACGAT AAGGAGACCT GCTTTGCCGA GGAGGGTAA	AAACTTGTG CTGCAAGTCA	1740
	AGCTGCCCTTA GGCTTATAAC ATCTACATT AAAAGCATCT	CAGCCTACCA TGAGAATAAG	1800
	AGAAAGAAAA TGAAGATCAA AAGCTTATTC ATCTGTTTC	TTTTCTGTTG GTGAAAGGCC	1860
	AACAC		1865

(2) INFORMATION FOR SEQ ID NO:13:

25 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 352 amino acids
 (B) TYPE: amino acid
 (C) TOPOLOGY: linear

30
(ii) MOLECULE TYPE: protein
(vii) IMMEDIATE SOURCE:
 (B) CLONE: native proBPN' amino acid sequence

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

	Ala	Gly	Lys	Ser	Asn	Gly	Glu	Lys	Tyr	Ile	Val	Gly	Phe	Lys	Gln	
	1					5			10					15		
40	Thr	Met	Ser	Thr	Met	Ser	Ala	Ala	Lys	Lys	Lys	Asp	Val	Ile	Ser	Glu
						20			25					30		
	Lys	Gly	Gly	Lys	Val	Gln	Lys	Gln	Phe	Lys	Tyr	Val	Asp	Ala	Ala	Ser
						35			40				45			
45	Ala	Thr	Leu	Asn	Glu	Lys	Ala	Val	Lys	Glu	Leu	Lys	Lys	Asp	Pro	Ser
						50			55			60				
	Val	Ala	Tyr	Val	Glu	Glu	Asp	His	Val	Ala	His	Ala	Tyr	Ala	Gln	Ser
						65			70			75			80	
	Val	Pro	Tyr	Gly	Val	Ser	Gln	Ile	Lys	Ala	Pro	Ala	Leu	His	Ser	Gln
						85			90			95				
50	Gly	Tyr	Thr	Gly	Ser	Asn	Val	Lys	Val	Ala	Val	Ile	Asp	Ser	Gly	Ile
						100			105			110				
	Asp	Ser	Ser	His	Pro	Asp	Leu	Lys	Val	Ala	Gly	Gly	Ala	Ser	Met	Val
						115			120			125				
	Pro	Ser	Glu	Thr	Asn	Pro	Phe	Gln	Asp	Asn	Asn	Ser	His	Gly	Thr	His
						130			135			140				
55	Val	Ala	Gly	Thr	Val	Ala	Ala	Leu	Asn	Asn	Ser	Ile	Gly	Val	Leu	Gly
						145			150			155			160	
	Val	Ala	Pro	Ser	Ala	Ser	Leu	Tyr	Ala	Val	Lys	Val	Leu	Gly	Ala	Asp
						165			170			175				
60	Gly	Ser	Gly	Gln	Tyr	Ser	Trp	Ile	Ile	Asn	Gly	Ile	Glu	Trp	Ala	Ile
						180			185			190				
	Ala	Asn	Asn	Met	Asp	Val	Ile	Asn	Met	Ser	Leu	Gly	Gly	Pro	Ser	Gly
						195			200			205				
	Ser	Ala	Ala	Leu	Lys	Ala	Ala	Val	Asp	Lys	Ala	Val	Ala	Ser	Gly	Val
						210			215			220				
65	Val	Val	Val	Ala	Ala	Ala	Gly	Asn	Glu	Gly	Thr	Ser	Gly	Ser	Ser	Ser
						225			230			235			240	
	Thr	Val	Gly	Tyr	Pro	Gly	Lys	Tyr	Pro	Ser	Val	Ile	Ala	Val	Gly	Ala

	245	250	255
	Val Asp Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Val Gly Pro Glu		
	260	265	270
5	Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr Leu Pro Gly		
	275	280	285
	Asn Lys Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala Ser Pro His Val		
	290	295	300
	Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Asn Trp Thr Asn		
10	305	310	315
	Thr Gln Val Arg Ser Ser Leu Glu Asn Thr Thr Lys Leu Gly Asp		
	325	330	335
	Ser Phe Tyr Tyr Lys Gly Leu Ile Asn Val Gln Ala Ala Gln		
	340	345	350

15 (2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1056 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:

- (B) CLONE: native proBPN' coding sequence

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

	GCAGGGAAAT CAAACGGGGA AAAGAAAAT ATTGTCCGGGT TTAAACAGAC AATGAGCACG	60
	ATGAGCGCCG CTAAGAACGAA AGATGTCATT TCTGAAAAAG GCGGGAAAGT GCAAAAGCAA	120
30	TTCAAAATATG TAGACGCAGC TTCAGCTACA TTAAACGAAA AAGCTGTAAA AGAATTGAAA	180
	AAAGACCCGA CGCTCGCTTA CGTTGAAGAA GATCACGTAG CACATGCGTA CGCGCAGTCC	240
	GTGCCTTACG CGCTATCACA ATTAAAGCC CCTGCTCTGC ACTCTCAAGG CTACACTGGA	300
	TCAAATGTTA AAGTAGCGGT TATCGACAGC GGTATCGATT CTTCTCATCC TGATTTAAAG	360
	GTAGCAGGCG GAGCCAGCAT GGTTCCCTCT GAAACAAATC CTTTCCAAGA CAACAACTCT	420
35	CACGGAACTC ACGTTGCCGG CACAGTTGCG CTCCTTAATA ACTCAATTGG TGTTAGGC	480
	GTTGCCCAA GCGCATCACT TTACGCTGTA AAAGTTCTCG GTGCTGACGG TTCCGGCCAA	540
	TACAGCTGGA TCATTAACGG AATCGAGTGG GCGATCGCAA ACAATATGGA CGTTATTAAC	600
	ATGAGCCTCG GCGGACCTTC TGGTTCTGCT GCTTTAAAAG CGGCAGTTGA TAAAGCCGTT	660
40	GCATCCGGCG TCGTAGTCGT TGCGGCAGCC GGTAAACGAAG GCACTCCGG CAGCTCAAGC	720
	ACAGTGGGCT ACCCTGGTAA ATACCCTCT GTCATTGCAG TAGGCCGTGT TGACAGCAGC	780
	AACCAAAGAG CATCTTTCTC AAGCGTAGGA CCTGAGCTTG ATGTCATGGC ACCTGGCGTA	840
	TCTATCCAAA GCACGCTTCC TGGAAACAAA TACGGGGCGT ACAACGGTAC GTCAATGGCA	900
	TCTCCGCACG TTGCCGGAGC GGCTGCTTTG ATTCTTTCTA AGCACCCGAA CTGGACAAAC	960
45	ACTCAAGTCC GCAGCAGTT AGAAAACACC ACTACAAAAC TTGGTGATTTC TTTCTACTAT	1020
	GGAAAAGGGC TGATCAACGT ACAGGGGGCA GCTCAG	1056

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 77 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:

- (B) CLONE: subtilisin BPN' pro-peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

60	Ala Gly Lys Ser Asn Gly Glu Lys Tyr Ile Val Gly Phe Lys Gln	
	1 5 10 15	
	Thr Met Ser Thr Met Ser Ala Ala Lys Lys Lys Asp Val Ile Ser Glu	
	20 25 30	
65	Lys Gly Lys Lys Val Gln Lys Gln Phe Lys Tyr Val Asp Ala Ala Ser	
	35 40 45	
	Ala Thr Leu Asn Glu Lys Ala Val Lys Glu Leu Lys Lys Asp Pro Ser	
	50 55 60	

Val Ala Tyr Val Glu Glu Asp His Val Ala His Ala Tyr
65 70 75

5 (2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 275 amino acids
(B) TYPE: amino acid
10 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:

- (B) CLONE: native mature BPN' amino acid sequence

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Ala Gln Ser Val Pro Tyr Gly Val Ser Gln Ile Lys Ala Pro Ala Leu
1 5 10 15
20 His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys Val Ala Val Ile Asp
20 25 30
Ser Gly Ile Asp Ser Ser His Pro Asp Leu Lys Val Ala Gly Gly Ala
35 40 45
Ser Met Val Pro Ser Glu Thr Asn Pro Phe Gln Asp Asn Asn Ser His
25 50 55 60
Gly Thr His Val Ala Gly Thr Val Ala Ala Leu Asn Asn Ser Ile Gly
65 70 75 80
Val Leu Gly Val Ala Pro Ser Ala Ser Leu Tyr Ala Val Lys Val Leu
85 90 95
30 Gly Ala Asp Gly Ser Gly Gln Tyr Ser Trp Ile Ile Asn Gly Ile Glu
100 105 110
Trp Ala Ile Ala Asn Asn Met Asp Val Ile Asn Met Ser Leu Gly Gly
115 120 125
Pro Ser Gly Ser Ala Ala Leu Lys Ala Ala Val Asp Lys Ala Val Ala
35 130 135 140
Ser Gly Val Val Val Val Ala Ala Gly Asn Glu Gly Thr Ser Gly
145 150 155 160
Ser Ser Ser Thr Val Gly Tyr Pro Gly Lys Tyr Pro Ser Val Ile Ala
165 170 175
40 Val Gly Ala Val Asp Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Val
180 185 190
Gly Pro Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr
195 200 205
Leu Pro Gly Asn Lys Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala Ser
45 210 215 220
Pro His Val Ala Gly Ala Ala Leu Ile Leu Ser Lys His Pro Asn
225 230 235 240
Trp Thr Asn Thr Gln Val Arg Ser Ser Leu Glu Asn Thr Thr Lys
245 250 255
50 Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val Gln Ala
260 265 270
Ala Ala Gln
275

55 (2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 275 amino acids
(B) TYPE: amino acid
60 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:

- (B) CLONE: amino acid sequence of mature BPN' variant

65

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Ala Gln Ser Val Pro Tyr Gly Val Ser Gln Ile Lys Ala Pro Ala Leu
 1 5 10 15
 His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys Val Ala Val Ile Asp
 20 25 30
 Ser Gly Ile Asp Ser Ser His Pro Asp Leu Lys Val Ala Gly Gly Ala
 35 40 45
 10 Ser Met Val Pro Ser Glu Thr Asn Pro Phe Gln Asp Thr Asn Ser His
 50 55 60
 Gly Thr His Val Ala Gly Thr Val Ala Ala Leu Thr Asn Ser Ile Gly
 65 70 75 80
 Val Leu Gly Val Ala Pro Ser Ala Ser Leu Tyr Ala Val Lys Val Leu
 85 90 95
 15 Gly Ala Asp Gly Ser Gly Gln Tyr Ser Trp Ile Ile Asn Gly Ile Glu
 100 105 110
 Trp Ala Ile Ala Asn Asn Met Asp Val Ile Thr Met Ser Leu Gly Gly
 115 120 125
 20 Pro Ser Gly Ser Ala Ala Leu Lys Ala Ala Val Asp Lys Ala Val Ala
 130 135 140
 Ser Gly Val Val Val Val Ala Ala Ala Gly Asn Glu Gly Thr Ser Gly
 145 150 155 160
 Ser Ser Ser Thr Val Gly Tyr Pro Gly Lys Tyr Pro Ser Val Ile Ala
 165 170 175
 25 Val Gly Ala Val Asp Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Val
 180 185 190
 Gly Pro Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr
 195 200 205
 30 Leu Pro Gly Asn Lys Tyr Gly Ala Tyr Ser Gly Thr Ser Met Ala Ser
 210 215 220
 Pro His Val Ala Gly Ala Ala Leu Ile Leu Ser Lys His Pro Thr
 225 230 235 240
 Trp Thr Asn Thr Gln Val Arg Ser Ser Leu Glu Asn Thr Thr Thr Lys
 245 250 255
 35 Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val Gln Ala
 260 265 270
 Ala Ala Gln
 275

40 (2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1260 base pairs
 45 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- (vii) IMMEDIATE SOURCE:
 50 (B) CLONE: codon-optimized 3D signal peptide-AAT DNA sequence

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

ATGAAGAACCA CCTCCTCCCT CTGCCCTCGT CGCTCGTGG TCCTCTGCTC CCTGACCTGC 60
 55 AACAGCGGCC AGGCCGAGGA CCCGAGGGC GACGCCGCC AGAACCGA CACCAGCCAC 120
 CACGACAGG ACCACCCGAC GTTCAACAAG ATCACCCCCA ATTGGCCGA ATTGGCCCTTC 180
 AGCCTGTACC GCCAGCTCGC GCACCAAGTCC AACTCCACCA ACATCTTCTT CAGCCGGTG 240
 AGCATCGCCA CCGCCTCGC CATGCTGTCC CTGGGTACCA AGGCGGACAC CCACGACGAG 300
 ATCCTCGAAG GGCTGAACCT CAACCTGACG GAGATCCCAGG AGGCGCAGAT CCACGAGGGC 360
 60 TTCCAGGGAC TGCTCAGGAC GCTCAACCAG CCGGACTCCC AGCTCCAGCT CACCACCGGC 420
 AACGGGCTCT TCCGTCCGA GGGCCTCAAG CTCGTCGATA AGTTCTGGGA GGACGTGAAG 480
 AAGCTCTACC ACTCCGAGGC GTTCACCGTC AACTTCGGGG ACACCGAGGA GGCCAAGAAAG 540
 CAGATCAACG ACTACGTGCA GAAGGGGACCC CAGGGCAAGA TCGTGGACCT GGTCAAGGAA 600
 65 TTGGACAGGG ACACCGTCTT CGCGCTCGTC AACTACATCT TCTTCAAGGG CAAGTGGGAG 660
 CGCCCGTTCG AGGTGAAGGA CACCGAGGAG GAGGACTTCC ACGTCGACCA GGTCAACCACC 720
 GTCAAGGTCC CGATGATGAA GAGGCTCGGC ATGTTCAACA TCCAGCACTG CAAGAAGCTC 780
 TCCAGCTGGG TGCTCCTCAT GAAGTACCTG GGGAACGCCA CCGCCATCTT CTTCCCTGCCG 840

	GACGAGGGCA AGCTCCAGCA CCTGGAGAAC GAGCTGACGC ACGACATCAT CACGAAGTTC	900
	CTGGAGAACG AGGACAGGGC CTCCGCTAGC CTCCACCTCC CGAAGCTGAG CATCACCGGC	960
	ACGTACGACC TGAAGAGCGT GCTGGGCCAG CTGGGCATCA CGAAGGTCTT CAGCAACGGC	1020
5	CGGGACCTCT CGCGCGTGCAG GGAGGAGGCC CCCCTGAAGC TCTCCAAGGC CGTGCACAAG	1080
	GCGGTGCTCA CGATCGACGA GAAGGGGACG GAAGCTGCCG GGGCCATGTT CCTGGAGGCC	1140
	ATCCCCATGT CCATCCCAGC CGAGGTCAAG TTCAACAAGC CCTTCGTCTT CCTGATGATC	1200
	GAGCAGAACCGA CGAAGAGCCC CCTCTCATG GGGAAAGGTG TCAAACCCAC GCAGAAAGTGA	1260

(2) INFORMATION FOR SEQ ID NO:19:

- 10 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1382 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- 15 (vii) IMMEDIATE SOURCE:
 (B) CLONE: codon-optimized 3D signal peptide-ATIII DNA sequen

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

	ATGAAGAACACCTCCTCCCT CTGCCTCCTG CTGCTCGTGG TCCTCTGCTC CCTGACCTGC	60
	AACAGCGGCC AGGCCACGG AAGCCCTGTG GACATCTGCA CAGCCAAGCC GCGGGACATT	120
	CCCATGAATC CCATGTGCAT TTACCGCTCC CGCGAGAAGA AGGCAACTGA GGATGAGGGC	180
25	TCAGAACAGA AGATCCCGGA GGCCACCAAC CGGCGTGTCT GGGAACTGTC CAAGGCCAAT	240
	TCCCCGTTTG CTACCACTTT CTATCAGCAC CTGGCAGATT CCAAGAATGA CAATGATAAC	300
	ATTTTCTGT CACCCCTGAG TATCTCCACG GCTTTTGCTA TGACCAAGCT GGGTGCCTGT	360
	AATGACACCC TCCAGCAACT GATGGAGGTA TTTAAGTTTG ACACCATATC TGAGAAAACAA	420
	TCTGATCAGA TCCACTTCTT CTGGCCAACTGCTGAACTGCC GACTCTATCG AAAAGCCAAC	480
30	AAATCCTCCA AGTAGTATC AGCCAACTGC CTTTTGGAG ACAAACTCCCT TACCTTCAAT	540
	GAGACCTACC AGGACATCAG TGAGTTGGTA TATGGAGCCA AGCTCAGGCC CCTGGACTTC	600
	AAGGAAATG CAGAGCAATC CAGAGCGGCC ATCAACAAAT GGGTGTCCAA TAAGACCGAA	660
	GGCCGAATCA CCGATGTCA TCCCTCGGAA GCCATCAATG AGCTCACTGT TCTGGTGCTG	720
	GTAAACACCA TTTACTTCAA GGGCTGTGG AAGTCAAAGT TCAGCCCTGA GAACACAAGG	780
35	AAGGAACGTG TCTACAAGGC TGATGGAGAG TCGTGTTCAG CATCTATGAT GTACCAGGAA	840
	GGCAAGTTCC GTTATCGGGC CGTGGCTGAA GGCACCCAGG TGCTTGAGTT GCCCTTCAA	900
	GGTGATGACA TCACCATGGT CCTCATCTTG CCCAAGCCCT AGAAGAGCCT GGCCAAGGTG	960
	GAGAAGGAAC TCACCCCGAGA GGTGCTGCAG GAGTGGCTGG ATGAATTGGA GGAGATGATG	1020
	CTGGTGGTTACATGCCCG CTTCCGCATT GAGGACGGCT TCAGTTGAA GGAGCAGCTG	1080
40	CAAGACATGG GCCTTGTGCA TCTGTTTCAGC CCTGAAAGAT CCAAACATCCC AGGTATTGTT	1140
	GCAGAAAGGCC GAGATGACCT CTATGCTCA GATGCATTCC ATAAGGCATT TCTTGAGGTA	1200
	AATGAAGAACG GCACTGAAAG AGCTGCAAGT ACCGCTGTG TGATTGCTGG CGITTCGCTA	1260
	AACCCAACA GGGTACTTT CAAGGCCAAC AGGCCCTTC TGTTTTTAA AAGAGAAGTT	1320
	CCTCTGAACA CTATTATCTT CATGGCAGA GTAGCCAACC CTTGTGTTAA GTAACATCGAG	1380
45	CC	1382

(2) INFORMATION FOR SEQ ID NO:20:

- 50 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1940 base pairs.
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- 55 (vii) IMMEDIATE SOURCE:
 (B) CLONE: codon-optimized 3D signal peptide-HSA DNA sequence

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

60	ATGAAGAACACCTCCTCTGCTG CTGCTCGTGG TCCTCTGCTC CCTGACCTGC	60
	AACAGCGGCC AGGCCAGATG CACACAAGAG TGAGGTTGCT CATGGTTTA AAGATTTGGG	120
	AGAAGAAAAT TTCAGAACCT TGTTGTTGAT TGCCTTTGCT CAGTATCTTC AGCAGTGTCC	180
	ATTTGAAGAT CATGAAAAT TAGTGAATGA AGTAACGAA TTTGCAAAAAA CATGTGTAGC	240
	TGATGAGTCA GCTGAAAATT GTGACAAATC ACTTCATACC CTTTTGGAG ACAAAATTATG	300
65	CACAGTTGCA ACTCTTCGTG AAACCTATGG TGAAATGGCT GACTGCTGTG CAAAACAAGA	360
	ACCTGAGAGA AATGAATGCT TCTTGCACAA CAAAGATGAC AACCCAAACC TCCCCCGATT	420
	GGTGAGACCA GAGGTTGATG TGATGTGCAC TGCTTTCAT GACAATGAAG AGACATTGTT	480

	GAAAAAAATAC TTATATGAAA TTGCCAGAAG ACATCCTTAC TTTTATGCCCGGAACTCCT	540
	TTCTTGT AAAAGGTATA AAGCTGCTT TACAGAATGT TGCCAAGCTG CTGATAAAGC	600
	TGCTGCCTG TTGCCAAAGC TCGATGAAC TCGGGATGAA GGGAAAGGCTT CGTCTGCCAA	660
5	ACAGAGACTC AAATGTGCCA GTCTCCAAA ATTGGAGAA AGAGCTTCA AAGCATGGGC	720
	AGTGGCTCGC CTGAGCCAGA GATTTCCCAA AGCTGAGTT GCAGAAGTTT CCAAGTTAGT	780
	GACAGATCTT ACCAAAGTCC ACACGGAAATG CTGCCATGGA GATCTGCTTG ATGTTGCTGA	840
	TGACAGGGCG GACCTTGCA AGTATATCTG TGAAAATCAG GATTGATCT CCAGTAAACT	900
	GAAGGAATGC TGTAACCTCTGGA AAAATCCCAC TGCATTGCCG AAGTGGAAA	960
10	TGATGAGATG CCTGCTGACT TGCTTCATT AGCTGCTGAT TTTGTTGAAA GTAAGGATGT	1020
	TTGCAAAAC TATGCTGAGG CAAAGGATGT CTTCCTGGGC ATGTTTTGT ATGAATATGC	1080
	AAGAAGGCAT CCTGATTACT CTGCTGCTG GCTGCTGAGA CTTGCCAAGA CATATGAAAC	1140
	CACTCTAGAG AAGTGCTGTG CCGCTGCAGA TCCTCATGAA TGCTATGCCA AAGTGGTCGA	1200
	TGAATTAAA CCTCTTGTGG AAGAGCCTCA GAATTAAATC AAACAAAATC GTGAGCTTT	1260
15	TAAGCAGCTT GGAGAGTACA AATTCCAGAA TGCGCTATTAA GTTCGTTACA CCAAGAAAGT	1320
	ACCCCAAGTG TCAACTCCAA CTCTTGAGA GGTCTCAAGA AACCTAGGAA AAGTGGGCAG	1380
	CAAATGTTGT AAACATCCTG AAGCAAAAG AATGCCCTGT GCAGAAGACT ATCTATCCGT	1440
	GGTCCTGAAC CAGTTATGTG TGTTGCATGA GAAAACGCCA GTAAGTGACA GAGTCACAAA	1500
	ATGCTGCACA GAGTCCTTGG TGAACAGGGC ACCATGCTTT TCAGCTCTGG AAGTCGATGA	1560
20	AACATACGTT CCCAAAGAGT TTAATGCTGA AACATTCAACC TTCCATGCAG ATATATGCAC	1620
	ACTTTCTGAG AAGGAGAGAC AAATCAGAA ACAAACTGCA CTTGTTGAGC TTGTGAAACA	1680
	CAAGCCAAG GCAACAAAG AGCAACTGAA AGCTGTTATG GATGATTTCG CAGTTTTGT	1740
	AGAGAAGTGC TGCAAGGCTG ACGATAAGGA GACCTGCTTT GCCGAGGAGG GTAAAAAAACT	1800
	TGTTGCTGCA ACTCAAGCTG CCTTAGGCTT ATAACATCTA CATTAAAAG CATCTCAGCC	1860
25	TACCATGAGA ATAAGAGAAA GAAAATGAAG ATCAAAAGCT TATTGATCTG TTTTCTTTT	1920
	CGTTGGTGTAAAGCCAACAC	1940

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1140 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- 35 (vii) IMMEDIATE SOURCE:
 - (B) CLONE: codon-optimized 3D signal peptide-BPN' DNA sequence

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

40	ATGAAGAACACCTCCTCCCTCTGCCCTCTGCTGCTGCTGGTCCCTCTGCTCCTGACCTGC	60
	AACAGCGGCCAGGCCGCTGGCAAGAGCACGGGGAGAAGAGTACATCGTCCGGCTTCAG	120
	CAGACCATGAGCACCAGATGAGCGCCGCCAAGAAGAGGACGTCATCAGCGAAGAGGGCGC	180
	AAGGTACAGAGCAGTTCAA GTACCTGGACGCCGCGCAGCGCCACCTCAA CGAGAAGGCC	240
	GTCAAGGAGCTGAAGAAGGCCGAGCGTCGCCCTACGTCAGGAGACCGTCGCCAC	300
45	GCATATGACAGAGCGTCCGTACGGCGTCAGCCAGATCAAGGCCCGGGCCTCCACAGC	360
	CAGGGCTACACCGGCAGCAA CGTCAAGGTCGCCGTACATCGACAGCGGCATCGACAGCAGC	420
	CACCCGGACC TCAAGGTGCGCGGAGCTAGCATGGTCCCGAGCGAGACCAACCCGTT	480
	CAGGACACCAACAGCCATGGCACCCACGTCGCCGGCACCGTCGCCGCCCTCACCAACAGC	540
	ATCGCGTCC TCGCGTCCGCCCGAGCGCCAGCCTCTACGCGTCAAGGTACTCGGCC	600
50	GACGGCAGCGGCCAGTACAGCTGGATCATCACGGCATCGAGTGGGCCATCGCCAAACAC	660
	ATGGACGTCA TCACCATGAGCTCGCGGCCCGAGCGGCCAGGCCGCCCTCAAGGGCGCC	720
	GTCGACAAGGCCGTCAGCGCGCTCGTCGCGCCGGCGCAA CGAGGGCACC	780
	AGCGGCAGCA GCAGCACCGTGGCTACCCGGGCAAGTACCGAGCGTCATGCCGTGCG	840
	GCCGTGGACA GCAGCAACCA GCGCGCGAGCTCAGCAGCTGGGCCATCGCC	900
55	ATGGCCCCGGCGTCAAGCATCCAGAGCACCCTCGGGCAACAAGTACGGCGCCTACAGC	960
	GGCACCCAGCA TGGCCAGCCC GCACCTCGCCGGCGCGCTGCACTCATCCTCAGCAAGCAC	1020
	CCGACCTGGACCAACACCCAGTCCCGCAGCAGCCTGGAGAACACCAACACCAACAGCTCGGC	1080
	GACAGCTTCTACTACGGCAA GGGCCTCATCACGTCCAGGCCCGCCAGTGACTCGAG	1140

60 (2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 13 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

- 65 (ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: N-terminus of mature AAT

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

5 Glu Asp Pro Gln Gly Asp Ala Ala Gln Lys Thr Asp Thr
1 5 10

(2) INFORMATION FOR SEQ ID NO:23:

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
15 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

20 GCTTGACCTG TAACTCGGGC CAGGCGAGCT

30

(2) INFORMATION FOR SEQ ID NO:24:

25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
30 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CGCCTAGCCC GAGTTACAGG TCAAGCAGCT

30

(2) INFORMATION FOR SEQ ID NO:25:

35 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
40 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

45 AGCTCCATGG CGGTGGCTCG AGTCTAGACG CGTCCCC

37

(2) INFORMATION FOR SEQ ID NO:26:

50 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
55 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

60 GGGGACGCGT CTAGACTCGA GCCACGGCCA TGG

33

(2) INFORMATION FOR SEQ ID NO:27:

65 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GCATGCAGGT GCTGAACACC ATGGTGAACA AACAC

35

5

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

TTCTTGTCCC TTTCGGT CCT CATGTCCTC CT

32

20

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

30

TGGCCTCTCC TCCAAC TTGA CAGCCGGGAG CT

32

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

TTCACCATGG TGTTCA GGCAC CTGCATGCTG CA

32

45

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

CGATGAGGAC CGAAAGGGAC AAGAAGTGTT TG

32

(2) INFORMATION FOR SEQ ID NO:32:

60

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

65

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CCCGGGCTGTC AAGTTGGAGG AGAGGCCAAG GAGGA

35

(2) INFORMATION FOR SEQ ID NO:33:

- 5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GAGGATCCCC AGGGAGATGC TGCCCAGAA

29

15

(2) INFORMATION FOR SEQ ID NO:34:

- 20 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CGCGCTCGAG TTATTTTG GG GTGGGATTCA CCAC

34

IT IS CLAIMED:

1. A method of producing, in monocot plant cells, a mature heterologous protein selected from the group consisting of

5 (i) mature, glycosylated α_1 -antitrypsin (AAT) having the same N-terminal amino acid sequence as mature AAT produced in humans and a glycosylation pattern which increases serum halflife substantially over that of mature non-glycosylated AAT;

(ii) mature, glycosylated antithrombin III (ATIII) having the same N-terminal amino acid sequence as mature ATIII produced in humans;

10 (iii) mature human serum albumin (HSA) having the same N-terminal amino acid sequence as mature HSA produced in humans and having the folding pattern of native mature HSA as evidenced by its bilirubin-binding characteristics; and

(iv) mature, active subtilisin BPN' (BPN') having the same N-terminal amino acid sequence as BPN' produced in *Bacillus*;

15 the method comprising:

(a) obtaining monocot cells transformed with a chimeric gene having (i) a monocot transcriptional regulatory region, inducible by addition or removal of a small molecule, or during seed maturation, (ii) a first DNA sequence encoding the heterologous protein, and (iii) a second DNA sequence encoding a signal peptide, said first and second DNA sequences in translation-frame 20 and encoding a fusion protein, and wherein (i) the transcriptional regulatory region is operably linked to the second DNA sequence, and (ii) said signal peptide is effective to facilitate secretion of the mature heterologous protein from the transformed cells;

(b) cultivating the transformed cells under conditions effective to induce said transcriptional regulatory region, thereby promoting expression of the fusion protein and secretion of the mature 25 heterologous protein from the transformed cells; and

(c) isolating said mature heterologous protein produced by the transformed cells.

2. The method of claim 1, wherein said first DNA sequence encodes proBPN', said cultivating includes cultivating said transformed cells at a pH between 5-6 to promote expression 30 and secretion of proBPN' from the cells, and said isolating step includes incubating the proBPN' under conditions effective to allow the autoconversion of proBPN' to active mature BPN'.

3. The method of claim 1, wherein said first DNA sequence encodes mature BPN', and said method further includes:

35 transforming said cells with a second chimeric gene containing (i) a transcriptional

regulatory region inducible by addition or removal of a small molecule, or during seed maturation, (ii) a third DNA sequence encoding the pro-peptide moiety of BPN', and (iii) a fourth DNA sequence encoding a signal polypeptide, where said fourth DNA sequence is operably linked to said transcriptional regulatory region and said third DNA sequence, and where said signal polypeptide is in translation-frame with said pro-peptide moiety and is effective to facilitate secretion of expressed pro-peptide moiety from the transformed cells;

5 said cultivating step includes cultivating the transformed cells at a pH between 5-6 to promote expression and secretion of BPN' and the pro-peptide moiety from the cells;

10 and said isolating step includes incubating the BPN' and the pro-moiety under conditions effective to allow the conversion of BPN' to active mature BPN', and isolating the active mature BPN'.

4. The method of claim 1, wherein said signal peptide is the RAmy3D signal peptide having the amino acid sequence identified by SEQ ID NO:1.

15

5. The method of claim 1, wherein said second DNA sequence encodes the RAmy3D signal peptide (SEQ ID NO:1) and has the codon-optimized nucleotide sequence identified by SEQ ID NO:3.

20

6. The method of claim 1, wherein said signal peptide is the RAmy1A signal peptide having the amino acid sequence identified by SEQ ID NO:4.

25

7. The method of claim 1, wherein the second DNA sequence, the first DNA sequence, or both the second and the first DNA sequence, is codon-optimized for enhanced expression in said plant.

30

8. The method of claim 1, wherein said transcriptional regulatory region is a promoter derived from a rice or barley α -amylase gene selected from the group consisting of the RAmy1A, RAmy1B, RAmy2A, RAmy3A, RAmy3B, RAmy3C, RAmy3D, and RAmy3E, pM/C, gKAmy141, gKAmy155, Amy32b, and HV18 genes.

35

9. The method of claim 8, wherein the chimeric gene further comprises, between said transcriptional regulatory region and said second DNA coding sequence, the 5' untranslated region of an inducible monocot gene selected from the group consisting of RAmy1A, RAmy3B, RAmy3C, RAmy3D, HV18, and RAmy3E.

10. The method of claim 8, wherein said chimeric gene further comprises, downstream of the sequence encoding said fusion protein, the 3' untranslated region of an inducible monocot gene derived from a rice or barley α -amylase gene selected from the group consisting of the RAmy1A, 5 RAmy1B, RAmy2A, RAmy3A, RAmy3B, RAmy3C, RAmy3D, and RAmy3E, pM/C, gKAmy141, gKAmy155, Amy32b, and HV18 genes.

11. The method of claim 1, wherein said cultivating includes culturing the transformed plant cells in a sugar-free or sugar-depleted medium, the transcriptional regulatory region is derived from 10 the RAmy3E or RAmy3D gene, the 5' untranslated region is derived from the RAmy1A gene and has the sequence identified by SEQ ID NO:5, and the 3' untranslated region is derived from the RAmy1A gene.

12. The method of claim 1, wherein the transformed cells are aleurone cells of mature 15 seeds, the transcriptional regulatory region is upregulated by addition of a small molecule to promote seed germination, and said cultivating includes germinating said seeds, either in embryonated or de-embryonated form.

13. The method of claim 12, wherein the transcriptional regulatory region is a rice α -amylose RAmy1A promoter or a barley HV18 promoter, and said small molecule is gibberellic acid. 20

14. A mature heterologous protein produced by the method of claim 1, wherein said protein is selected from the group consisting of:

(i) mature glycosylated α_1 -antitrypsin (AAT) having the same N-terminal amino acid 25 sequence as mature AAT produced in humans and having a glycosylation pattern which increases serum halflife substantially over that of non-glycosylated mature AAT;

(ii) mature glycosylated antithrombin III (ATIII) having the same N-terminal amino acid sequence as mature ATIII produced in humans; and

(iii) mature glycosylated subtilisin BPN' (BPN') having the same N-terminal amino acid 30 sequence as BPN' produced in *Bacillus*;

wherein said protein has a glycosylation pattern characteristic of proteins produced in said monocot plant.

15. The method of claim 1, wherein said monocot plant cells are transformed rice, barley, 35 corn, wheat, oat, rye, sorghum, or millet cells.

16. The method of claim 1, wherein said monocot plant cells are transformed rice or barley cells.

5 17. Plant cells capable of producing the mature heterologous protein according to the method of claim 1, wherein said cultivating includes culturing the transformed plant cells in a sugar-free or sugar-depleted medium, the transcriptional regulatory region is derived from the RAmy3E or RAmy3D gene, the 5' untranslated region is derived from the RAmy1A gene and has the sequence identified by SEQ ID NO:5, and the 3' untranslated region is derived from the RAmy1A gene.

10

18. Seeds capable of producing the mature heterologous protein according to the method of claim 1, wherein said transformed cells are aleurone cells, the transcriptional regulatory region is upregulated by addition of a small molecule to promote seed germination, and said cultivating includes germinating said seeds, either in embryonated or de-embryonated form.

15

3D Signal Peptide

non-codon optimized ATG AAG AAC ACC AGC AGC TGT TGT TGT TGT AAC TCG GGC CAG GCG
 codon-optimized ATG AAG AAC ACC TCC TCC CTC CTC TGC AAC AGC TGC ACC TCC CTC CTC GTC CAG GCC
 amino acid sequence Met Lys Asn Thr Ser Ser Leu Cys Leu Leu Val Val Cys Ser Leu Thr Cys Asn Ser Gly Gln Ala

Fig. 1

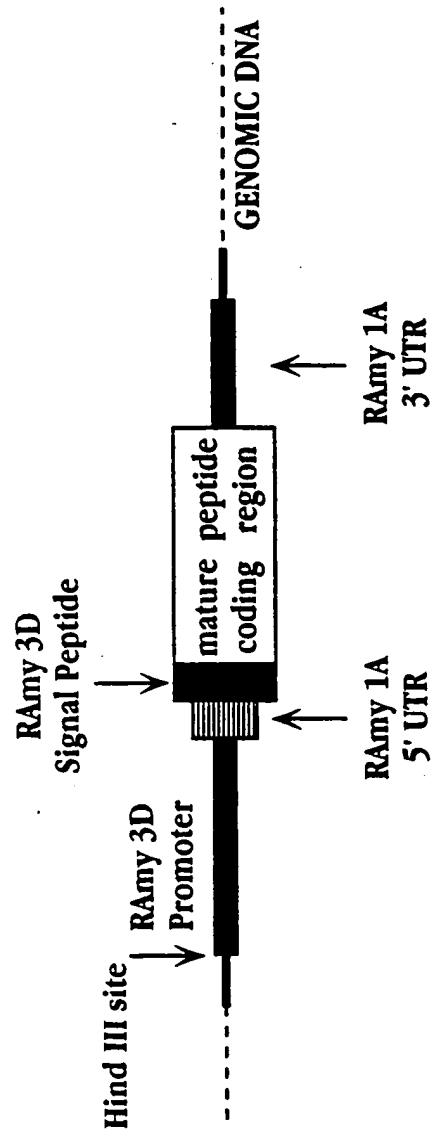
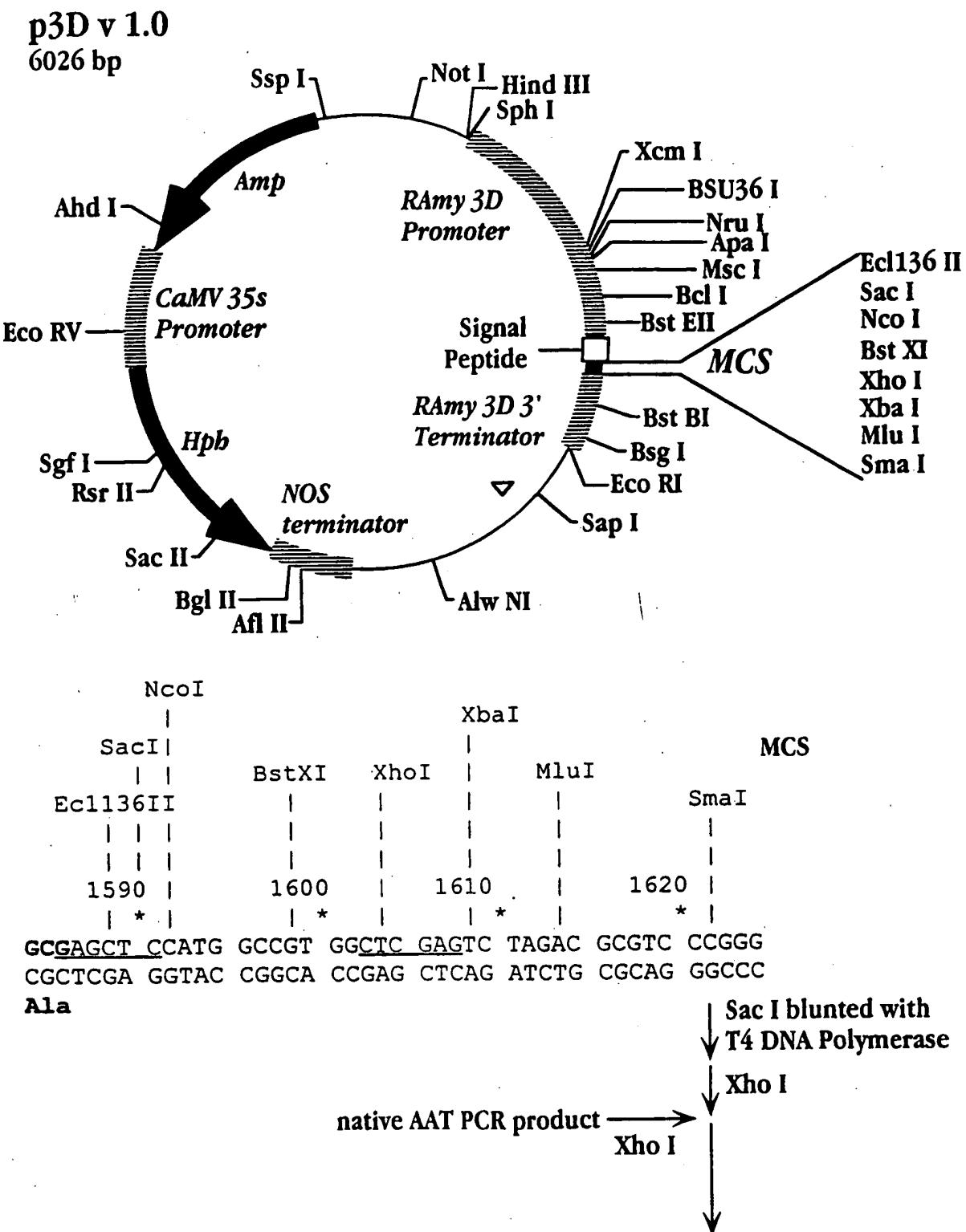


Fig. 2

**Fig. 3A**

p3D - AAT
7195 bp

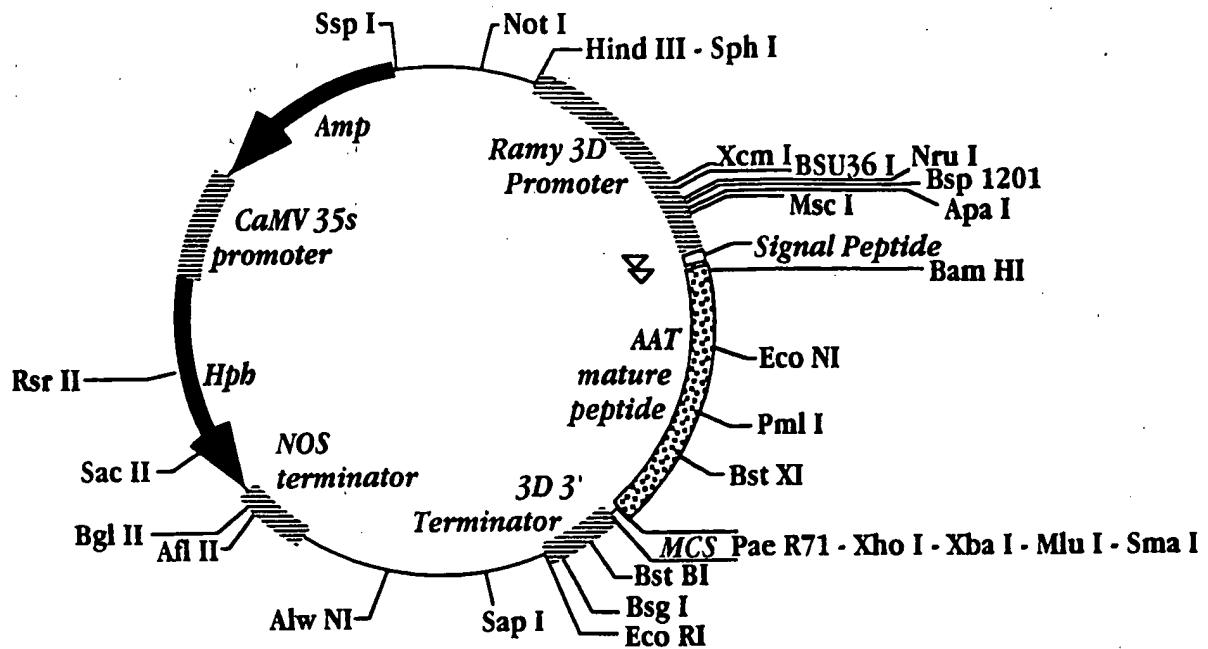


Fig. 3B

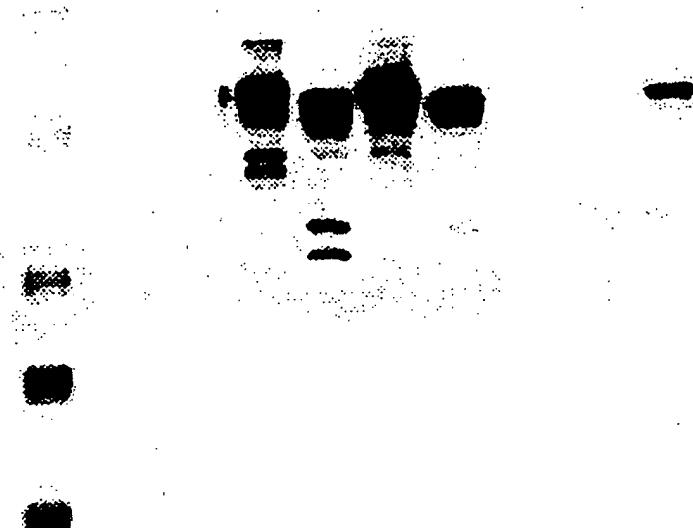
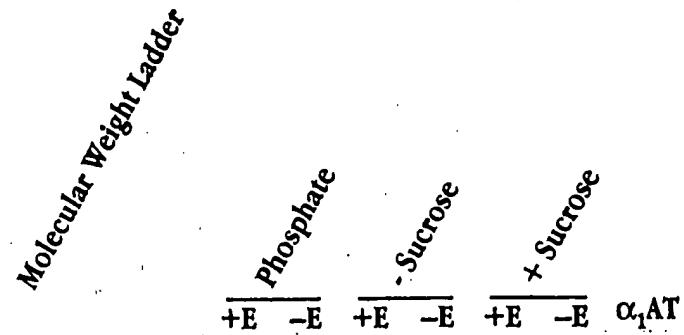


Fig. 4

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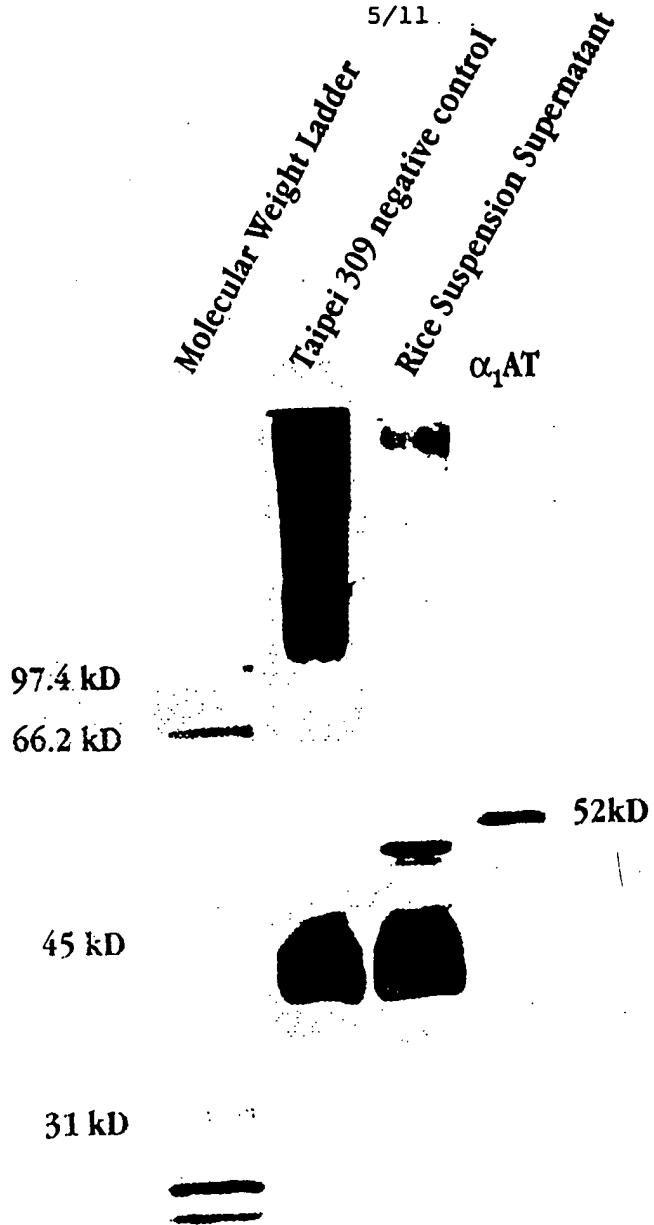


Fig. 5

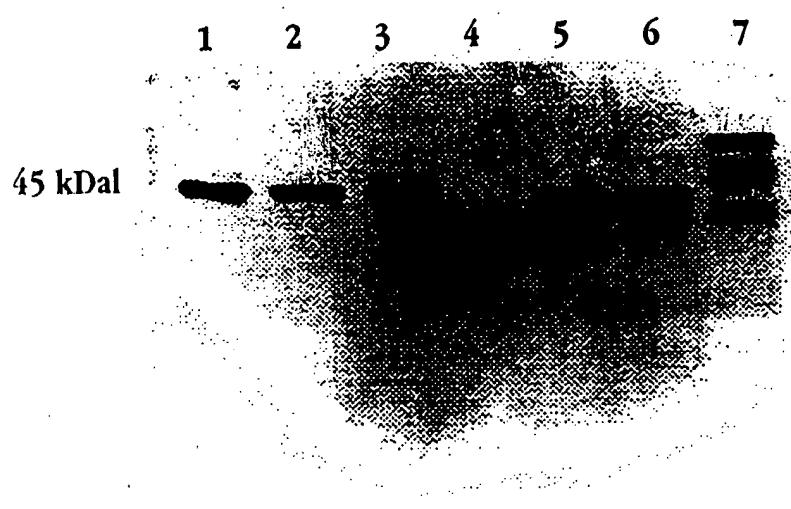


Fig. 6

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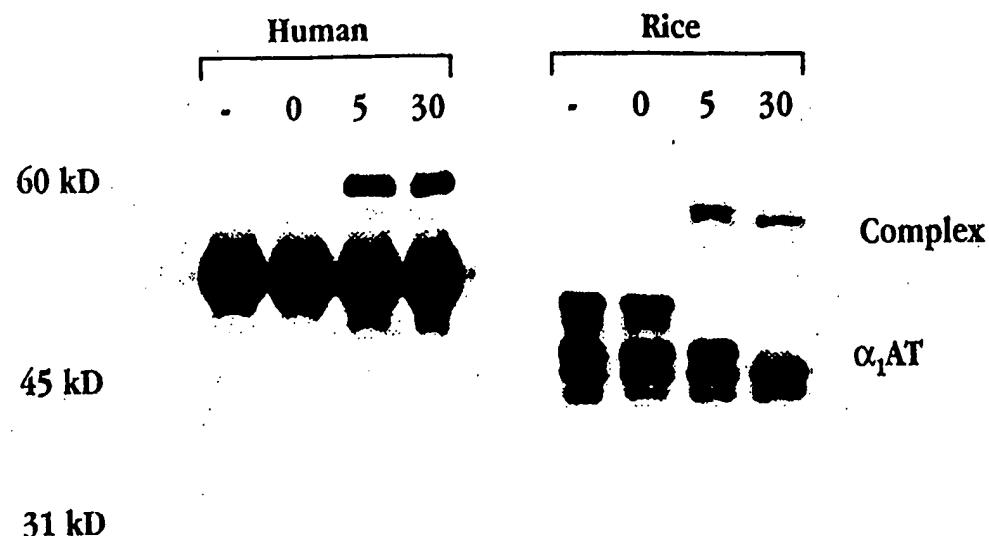


Fig. 7

N ----> E-D-P-Q-G-D-A-A-Q-K-T-D-T

Fig. 8

SUBSTITUTE SHEET (RULE 26)

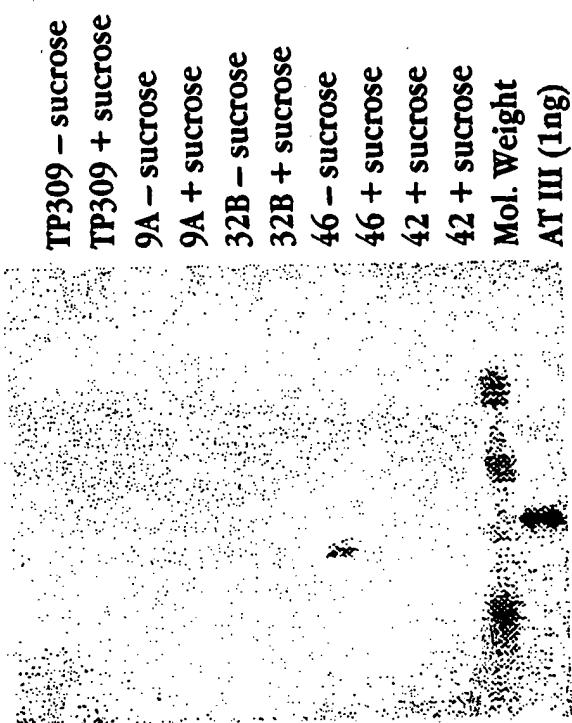


Fig. 9

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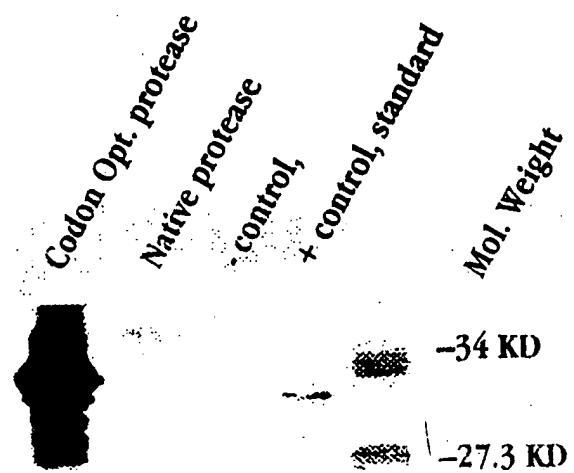
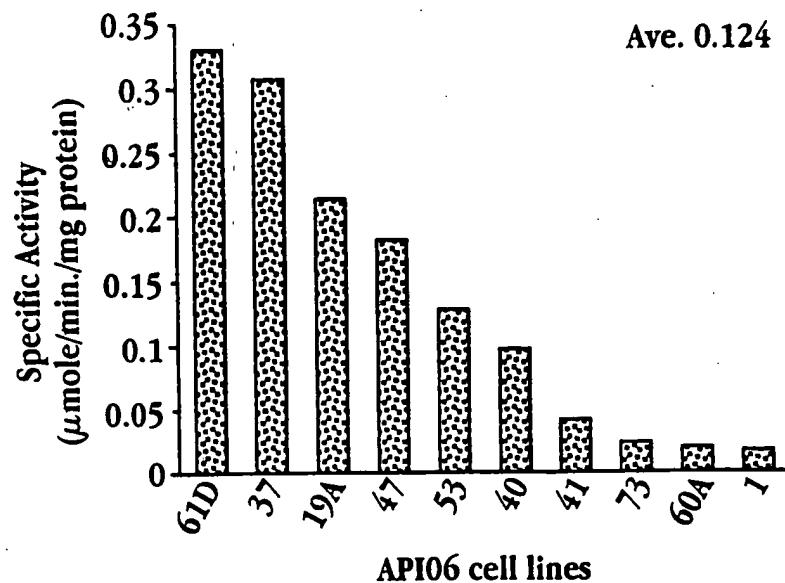
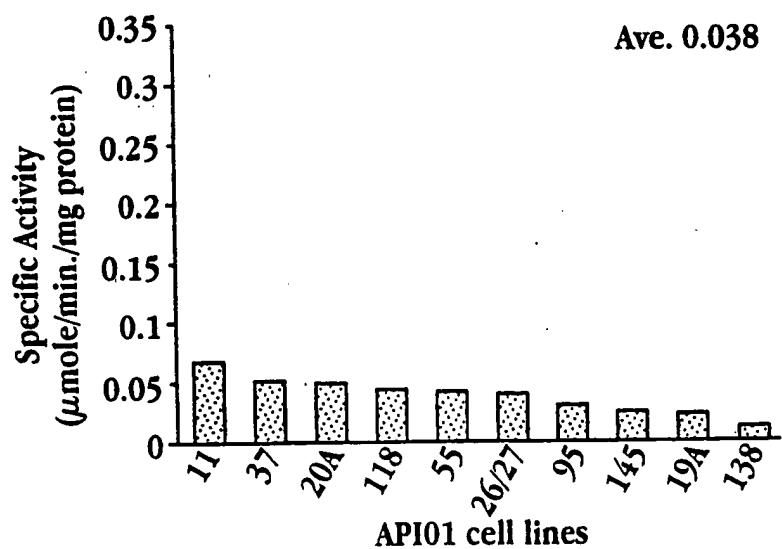


Fig. 10

**Fig. 11A****Fig. 11B**

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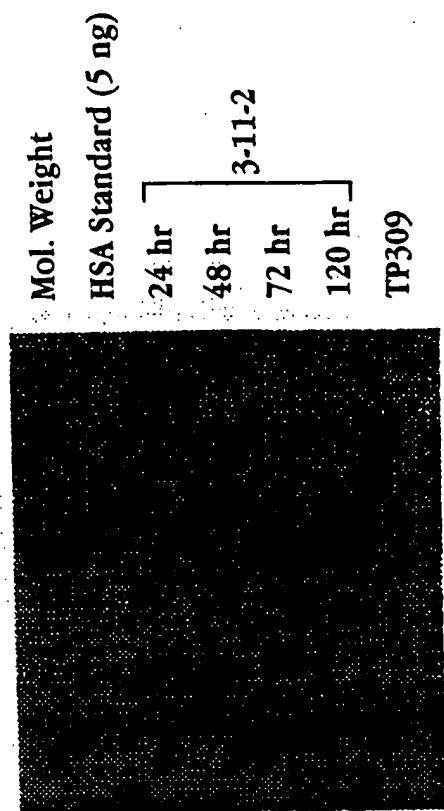


Fig. 12

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C12N15/82 C12N15/57 C12N15/15 C12N15/14 C12P21/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	WO 92 01042 A (NOVONORDISK AS) 23 January 1992 see page 6, line 15 - line 19 ---	1, 5
Y	JENSEN L G ET AL: "TRANSGENIC BARLEY EXPRESSING A PROTEIN-ENGINEERED, THERMOSTABLE (1,3-1,4)-BETA-FLUCANASE DURING GERMINATION" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 93, no. 8, April 1996, pages 3487-3491, XP002024710 see the whole document ---	5
	-/-	

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

30 June 1998

Date of mailing of the international search report

14/07/1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.
Fax: (+31-70) 340-3016

Authorized officer

Maddox, A

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>TERASHIMA M ET AL: "Production of functional human alpha-1-antitrypsin by rice cell culture; expression and protein secretion in callus culture (conference abstract)"</p> <p>ABSTR.PAP.AM.CHEM.SOC.;(1997) 214 MEET., PT.1, AGFD018 CODEN: ACSRAL ISSN: 0065-7727 AMERICAN CHEMICAL SOCIETY, 214TH ACS NATIONAL MEETING, LAS VEGAS, NV, 7-11 SEPTEMBER, 1997., XP002069835</p> <p>see abstract 018</p> <p>---</p>	1,4, 8-10, 14-17
A	<p>THOMAS, B. R. ET AL: "Gene regulation and protein secretion from plant cell cultures: the rice alpha - amylase system"</p> <p>ADVANCES IN PLANT BIOTECHNOLOGY, (1994)</p> <p>PP. 37-55. STUDIES IN PLANT SCIENCE 4. 85</p> <p>REF. PUBLISHER: ELSEVIER SCIENCE.</p> <p>AMSTERDAM ISBN: 0-444-89939-1, XP002069833</p> <p>see the whole document</p> <p>---</p>	1,11
A	<p>CHAN M-T ET AL: "Novel gene expression system for plant cells based on induction of alpha-amylase promoter by carbohydrate starvation."</p> <p>JOURNAL OF BIOLOGICAL CHEMISTRY 269 (26). 1994. 17635-17641. ISSN: 0021-9258,</p> <p>XP002069821</p> <p>see the whole document</p> <p>---</p>	1,11
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A	<p>EP 0 348 348 A (CIBA GEIGY AG) 27 December 1989</p> <p>see examples 41-45</p> <p>---</p>	1

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			IL	90640 A	04-01-1998
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